

What bright prosperity for Europe?

On a gloomy reading of recent trends, the European Union may never be able to deliver the rising prosperity that is its chief justification. The best hope is a stroke of luck with innovation.

CHILLING visions sometimes emerge from leisured and almost academic discussions. Thus it was at the conference last week in Paris on a strategy for research in Europe (jointly organized by the Paris-based Observatoire des Sciences et Techniques, the Science Policy Research Unit at the University of Sussex and *Nature*), of which a fuller report will appear in a later edition. Of necessity, speakers were concerned with the pattern of spending in research and development by European companies of various sizes, the articulation of perceived social needs with the planning of research and the nature of the innovation process. It was mostly a constructive, even upbeat, conversation.

But there is another side to that coin. There were repeated references last week to the beginning in Europe of the well-established practice of Japanese manufacturers of shifting production to places where labour costs are low, to continuing high unemployment in the European Union and to the prospect that continued pressure on public budgets will further reduce public spending on research just when the need for successful innovation is greater than ever. In passing, several speakers referred to the danger that social unrest would spring from the prolongation of unemployment and the frank deprivation now visible on European city streets. Yet everywhere it is taken as a given that European living standards must at least be maintained or, preferably, improved upon. The chilling question is whether that is now possible. May it not be, instead, that on the heels of the completion of the single market, and even before there is a European currency, Europe has already embarked on secular decline?

Among the wellsprings of this gloomy speculation, the most durable is that known as globalization, an awkward name for a complex of phenomena such as companies' relatively new-found freedom to invest in production capacity where they will, for the alarming truth that modern plants for making the more sophisticated products of high technology do not demand sophisticated production workers, but only sophisticated design, and for the truth that substantial parts of the markets for those sophisticated products are often in countries still anomalously regarded as developing countries.

Thus the legend of Japan's success with motor-cars in the past 25 years is now being replicated in South Korea. A large part of the explanation is that, in competition with several of the emerging economies, Europe has the built-in disadvantage of higher labour costs (which properly

account for the cost of employers' contributions to social security benefits and the like). On the average, labour costs are roughly 20 per cent greater than in the United States, 25 per cent greater than in Japan and roughly five times those in the fast-moving Asian economies. Given political stability in the Far East, the export of jobs from Europe will continue and the fight against unemployment will be against an adversely moving target.

The scale of the problem can be seen from the rate at which the so-called developing countries of the Far East have been growing. Between 1983 and 1992, gross domestic product (GDP) increased by 23 per cent in the European Union (EU), by 20 per cent in the United States, by 38 per cent in Japan but by 84 per cent in the 'tigers' of the Pacific rim (chiefly South Korea and Taiwan) and by no less than 102 per cent in China. These are once-and-for-all economic improvements that will not be cancelled out even if the rate of growth in Western Europe suddenly takes a turn for the better. But even that seems unlikely to happen quickly.

The pressure on public research funds had everybody depressed last week, even though the reasons are well understood. High unemployment (and the large social benefits it evokes) means that governments run fiscal deficits, causing inflation (or compelling them to follow deflationary policies) and putting their currencies at risk. In that respect, all European countries seem to be in the same boat. The pressure is made worse within the EU by the requirement of the Maastricht Treaty that members of the intended common currency regime should not run fiscal deficits exceeding 3 per cent of GDP at the time they join (in 1999). Only Germany and Luxembourg qualify as things are.

What is to be done? The magic wand would be an access of economic growth in Europe based largely on innovation rather than on the growth of industries that will in due course be exported to the Far East. That, no doubt, is why last week's meeting devoted much of its time to accounts of academic studies of the process, which is not to say that it is understood. True, there is more to successful innovation than a laboratory discovery followed by its industrial development, but the financial, political and even social circumstances that engender success were as elusive last week as at other meetings of the kind. On paper, a further transfer of research funds from national budgets to the centre at Brussels would bring economies of scale, but



there are two snags: European governments are far from persuaded that Brussels is a better custodian than they are themselves of scant research money, while this is a time when most governments are jealous of the doctrine of subsidiarity (that the centre should do only what national governments cannot). Europe is a long way from being the United States of Europe.

There are disturbing signs that Europe has not yet awakened to this gloomy prospect, or to the urgency with which it should somehow be forefended. It is conventional to speak of health and environmental quality as the benefits that European research will bring. The need that there should be a jump in economic growth comparable with that since, say, 1970, but concentrated into a decade, is in reality a more urgent social need. One of the early casualties of failure in this regard could easily be the European Union itself. □

Genome diversity alarms

The excellent Human Genome Diversity Project needs better planning and a pilot project.

PROFESSOR Luigi Cavalli-Sforza, the Stanford geneticist, has an excellent idea: to reconstruct the recent history of *Homo sapiens* from a comparison of the genomes of different human populations. Success with what is called the Human Genome Diversity Project (HGDP) could more quickly throw more light on this important question than anything likely to be forthcoming from palaeoanthropology in the near future. But there is also the intriguing likelihood that a combination of data from both sources may make it possible to identify the genetic correlates of particular physical human adaptations, thus throwing light on the malleability of the genome as an entity. Two cheers, in all the circumstances, for Cavalli-Sforza!

Why not three cheers? Because the HGDP has insufficiently anticipated the inevitable objections to its plans. To be sure, it has been endorsed as worthy (which it is) by the Human Genome Organization (HUGO), but that is a consortium of scientists. It is also true that many of the reasons offered at last week's meeting of the Unesco's Bioethics Committee (see page 373) for declining to support Cavalli-Sforza's plans are insubstantial. For example, the working group on population genetics almost perversely confused HGDP's plans with what it called eugenics in the sense of Hitler rather than, say, Francis Galton. Similarly, it gives undue weight to the fears that the genetic inheritance of indigenous people will be exploited commercially by "vampires" doing Cavalli-Sforza's bidding.

Yet there is a nub of reason in the decision. Put simply, a genetic comparison of distinct but isolated population groups must include a comparison of the genetic constitution of what are called racial groups. It is understandable that many people (and not only those directly involved) should be alarmed at the uses to which such information could be put, while researchers cannot guarantee that the

data they gather will be such that they cannot be misused; they can merely promise to use their best efforts to ensure that does not happen. Cavalli-Sforza himself says that experience so far shows that intra-group differences in physiological or psychological measures are always greater than the mean differences between groups, so that HGDP should help to combat racism. That misses the point of the objection, which is that the project may make racial members objectively identifiable, and to their disadvantage. So how should HGDP set out to win round those who doubt its motives?

The place to start is with the doctrine of informed consent, which applies to the engagement of individuals as subjects in research projects of all kinds, not just in human genetics. The purposes and procedures of the research must be fully explained, together with the objectives and the potential hazards. The understanding of the subjects must be so complete that they will be neither surprised nor disconcerted by the outcome of the projects in which they are engaged. They should agree to collaborate voluntarily. It is out of spirit with the doctrine of informed consent that people should be recruited to one project and that the same data or samples should be used for another (without informed consent to *that*). The principle of informed consent is excellent, and consistent with the "inherent dignity" of every person, to quote the United Nations' Universal Declaration of Human Rights (1948). The difficulty is that the extension of this principle to population groups is far from simple.

The difficulty is knowing who or what can speak for distinctive population groups. The population groups concerned are usually subgroups of national populations, but also often span frontiers. On anthropological grounds, it is estimated that there are about 5,000 population groups isolated from each other by language, geography and other factors, of which HGDP hopes to study 500. It could be imagined that the project could find islands in Polynesia or villages in New Guinea whose people would agree to give samples for the project in return for a tangible benefit of some kind, but that would offend against the voluntary principle, while the consent of a single community cannot be taken as consent by the whole of a population.

To make progress, HGDP should therefore plan to run a pilot project in circumstances in which these delicate issues would not arise, and where genetic knowledge is reasonably well spread. Almost any industrial country in Western Europe or North America would be a suitable substrate for an investigation. Data from such enquiries would bear only marginally on the grand questions of recent human evolution, but they would not be devoid of interest. Because the benefit for those taking part in the study would be that those carrying putative disease-linked alleles could be informed when appropriate and appropriately counselled, such a pilot study could help to demonstrate to indigenous people that genetic information can be beneficial and could also help to prove the counselling techniques that will be required if and when HGDP gets properly under way. □

Genetic diversity proposal fails to impress international ethics panel

Paris. A multimillion-dollar project to study genetic variation in populations worldwide — the Human Genome Diversity Project (HGD) — appears unlikely to receive the endorsement it has been seeking from the United Nations Educational Scientific and Cultural Organization (Unesco).

The project — in which DNA would be collected from around 25 individuals from about 500 of the 5,000 or so different ethnic groups — is the brainchild of Luigi Luca Cavalli-Sforza, a renowned population geneticist at Stanford University in California. Knowledge of the origins of populations would have “enormous potential for illuminating our understanding of human history and identity”, and provide information on the genetic basis of predisposition and resistance to disease.

The project, which it is estimated would cost around \$5 million annually, has been backed by the Human Genome Organization (HUGO). But it has obtained little funding, partly because of controversy about its procedures and implications. Its supporters have been lobbying Unesco to set up an ethics committee to oversee the project, in a bid to improve HGD's image and to increase its prospects for funding.

But at a meeting of Unesco's International Bioethics Committee (IBC) in Paris last week, a working group on population genetics set up by the committee strongly criticized the HGD, and recommended UN organizations not to endorse any individual project in this area in order to “safeguard” Unesco's “independence, neutrality and credibility”. One Unesco official also says it rejected a separate request from HDG for direct funding.

The working group acknowledged the

validity of the project's scientific goals. But it also endorsed criticisms by indigenous peoples, the main target of HGD. The group said, for example, that the project needs to clarify how — if at all — intellectual property rights would be claimed on biological material derived from populations.

Indigenous groups are angry that the US Department of Commerce has filed patent

Debra Harry, a Paiute North American Indian who works with the Indigenous People's Biodiversity Network, said that genetic research is “not a priority for indigenous peoples”. She pointed out that basic human rights, such as access to better health care, are a better guarantee of their well-being and survival, and added: “They've come to take our blood and tissues for their interests, not for ours.”

Harry invoked the 1964 Helsinki Declaration — “in research on man, the well-being of the subject takes precedence over science and society” — to argue for a halt to HGD on the grounds that indigenous peoples feel they will not benefit from it. Indeed, she claimed that information from HGD would in fact increase discrimination against indigenous peoples.

Cavalli-Sforza has argued that HGD would reduce the risk of racism by showing that the notion of race is flawed. But the IBC group described this as the “most debatable” claim of the project, arguing that the prejudice that gives rise to racist and eugenic attitudes tends to pervert scientific results to its own ends. Genetic reductionism, argued many at the meeting, represents a threat to those mythologies of human origins that are different from those of the dominant world cultures.

Moreover, the working group argues that opposition is based upon more than misunderstandings of the scientific aims or anti-science attitudes, but “is a clash of philosophy and cultural insight”. Harry, for example, says that “genetics is a violation of our ethics, it attacks our culture's world view”. She adds: “We don't view our genes as protein actions ready to be interpreted; for us our genes are sacred.”

One ethnological researcher at the meeting urged biologists to learn to “respect community rights”, adding that their discipline has become accustomed to working under enormous constraints. These include lengthy approval procedures, and making notes available to the groups being studied.

Cavalli-Sforza, who attended last week's meeting, said he welcomed the committee's analysis, and “shared their concerns”. HGD, he added, was drafting protocols that would go “even further” than the committee's recommendations.

But he dismissed the claims concerning the project's risks as “exaggerated”, and continued to attribute them to misunderstanding. “I have become used to being called a planner of genocide and of being accused of economic interest,” he says. “My main aim is to defend the project and defend science.”

Declan Butler

IMAGE
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REASONS

Natural rites: genome data 'could threaten traditional beliefs' of indigenous peoples.

claims on cell lines taken from indigenous people from the Solomon Islands. The islands' government has protested. But Ron Brown, the US Secretary of Commerce, replied last year that the origin of cells was irrelevant to the patent application.

The working group also asked HGD, which is managed by a subcommittee of HUGO, to “formulate concretely” its general claims that the project would benefit local populations. HGD's claim that it will lead to the development of local research laboratories, for example, needs to be clarified so that “it becomes obvious how this would happen”. The working group also wants confirmation that HGD would not seek commercial funding, despite its fragile financial situation, and called on HGD to include indigenous peoples in all stages of the project.

The long list of criticisms reflects a general feeling at the meeting that the enthusiasm of the project's supporters for scientific results has led to the neglect of wider issues, in particular human rights. The working group pointed out that although HGD has “expressed urgency” in collecting samples from peoples in danger of cultural and physical extinction, it had not expressed concern about their extinction *per se*.

Indeed, speaking at last week's meeting,

Medical charities win lottery funds

London. Britain's medical research charities could receive up to £50 million (US\$80 million) next year from the proceeds of the recently-launched national lottery, following complaints over their exclusion from the first round of charitable funds, due to be made later this month.

Many medical charities, particularly the relatively smaller ones which lack large legacies, say that they have suffered a significant drop in income since the lottery was launched. In announcing that the first distribution of grants next year will concentrate on health, disability and care, the National Lottery Charities Board is hoping to temper some of this criticism. □

US radiation report prompts bioethics move

Washington. A panel advising President Bill Clinton on radiation experiments conducted after World War Two has found "serious deficiencies" in the system for protecting human research subjects. Clinton was expected to announce the establishment of a National Bioethics Advisory Committee, to tackle the issues raised by the panel, when he received its final report on Tuesday.

In the report, the Advisory Committee on Human Radiation Experiments calls for a national effort "to ensure the centrality of ethics in the conduct of scientists whose research involves human subjects". It also recommends a shake-up of the system of non-specialist institutional review boards (IRBs) which has the task of maintaining research ethics.

But the panel concludes that the government does not owe any compensation to the thousands of unsuspecting subjects of the post-war radiation experiments, unless they were either physically harmed or had been systematically deceived. Experts said this ruling — if accepted by the administration and Congress — would probably restrict automatic compensation to a small number of subjects used in a handful of well-publicized experiments.

The report is the culmination of a huge, 18-month investigation that began when Hazel O'Leary, the energy secretary, promised to open up the records of the nuclear-related research undertaken by her department and its predecessors. With various degrees of reluctance, other branches of government then joined in a trawl through millions of documents on past radiation experiments (see *Nature* 368, 781; 1994).

According to several observers, the findings of the panel, which was chaired by Ruth Faden, a medical ethicist at Johns Hopkins University, Baltimore, Maryland, are much

more critical of existing research practice than had been expected. They follow a crucial decision of the panel, which was asked to focus chiefly on the period 1946–74, to check up on today's practice by conducting an extensive survey of 1,900 cancer and heart disease patients.

The report was welcomed, in public at least, by government officials responsible for upholding ethics in human subjects research. Gary Ellis, director of the Office for Protection from Research Risks (OPRR) at the National Institutes of Health (NIH), said that the panel had found "a well-rounded system", even though there was still some room for improvement. "We too have an interest in making the system better," he said.

Faden says that the panel did not intend to criticize the NIH office, which is "doing as much as it can with the resources it has — which are pitiful". OPRR, whose main function is to educate researchers, has 15 members of staff and an annual budget of \$2.2 million. This year, it has held five workshops for around 1,000 scientists, and it circulates a newsletter to 5,500 addressees.

The panel's criticism of current research practice focused on two main problems: the failure of many research subjects to understand properly what they are letting themselves in for, and the difficulties of non-specialist institutional review boards (IRBs) in adequately supervising research on human subjects.

"There is reason to worry that partici-



O'Leary: Initiated the review of experiments.

pants in research may have unrealistic expectations both about the possibility they will personally benefit from participation, and about the discomfort, pain and suffering that sometimes accompany research," says the report. "This seems particularly to be the case in Phase I and Phase II drug trials."

The panel calls for new mechanisms to ensure that patients have a better understanding of these risks and — in particular — grasp the distinction between research and treatment. It adds that the IRBs are overwhelmed with work, chiefly because they must assess every research grant proposal submitted to NIH, including the vast majority which go unfunded.

According to Faden, the panel's decision not to recommend automatic compensation for all patients subjected unknowingly to radiation experiments came after careful consideration of how a remedy should match an offence. The report suggests that these patients should get a formal apology from the federal government. Compensation should be paid only if the patient was harmed, or if the government actively sought to keep information from patients or families "for the purpose of avoiding embarrassment or legal liability".

The committee's intensive search of research records appears to have unearthed few instances of wantonly unethical conduct that had not already surfaced in the press, or when Congress investigated the subject ten years ago. Faden said that she was happy with the cooperation received from all government agencies, although the Central Intelligence Agency (CIA), she said, was "a special case". According to the report, the CIA's record-keeping practices and cellular structure made it impossible to get a complete picture of the research it conducted on human subjects.

The panel recommended a new federal policy to safeguard the right to informed consent for the human subjects of secret government research, which it said continued, albeit on a small scale. It found that the secret release of radiation into the atmosphere, for research purposes, remained legal, and said that this should be overseen by suitably cleared inspectors from the Environmental Protection Agency.

All of this will resonate with that sector of the public that instinctively distrusts the federal government. But it is likely also to annoy those researchers who believe that sufficient safeguards are already in place to prevent any repetition of past mistakes.

Faden agrees there has been "dramatic progress" in research ethics over the past 50 years. "So there is a temptation to say there was a problem, and we have fixed it," she says. But that would be a big mistake; "there are still serious problems and we need to keep working at them." **Colin Macilwain**

Omnibus science bill heads for the TV screen

Washington. Science policy issues will receive a thorough airing on Capitol Hill next week, when the House of Representatives considers an omnibus science bill outlining almost \$22 billion of spending on research and development.

The authorization bill, which is being proposed by Robert Walker (Republican, Pennsylvania), chair of the House science committee, covers most government-sponsored research and development, except biomedical and defence research. It deals with all research spending at the National Aeronautics and Space Administration (NASA), the National Science Foundation (NSF), the Department of Energy and the Department of Commerce.

Walker says that having a single bill for all those agencies will "elevate science to the same kind of consideration that defence pri-

orities have always had." The House is expected to allow two or three days from 10 October to debate the bill, giving US scientists with access to C-SPAN cable television a chance to see their paymasters at work.

The bill will propose cutting back next year's budget for these agencies from \$24.5 billion to \$21.5 billion. But Republicans say that the basic research component of that will hold steady at \$6.7 billion.

Democrats will try to restore various threatened budget items, including NASA's Mission to Planet Earth, the Department of Commerce's Advanced Technology Programme, and the Energy Department's conservation, solar energy and fusion programmes, and may even find some Republican allies. But the House is unlikely to overturn any major part of Walker's bill.

C. M.

Court charges open split in Greek earthquake experts

London. Leading Earth scientists in Greece have set up a committee to defend the director of the Greek earthquake planning and protection agency against charges of breach of duty for allegedly disregarding warnings of last June's magnitude 6.1 earthquake which killed 25 people.

Dimitris Papanikolaou has been accused by Athens' public prosecutor, Kolio Kostas, of ignoring two separate warnings of the earthquake. One was issued by Panagiotis Varotsos, professor of solid-state physics at the University of Athens, and the other by Gerasimos Papadopoulos, an associate professor at the Geodynamic Institute of the National Observatory of Athens.

Varotsos used his controversial VAN method, in which an impending earthquake is forecast from a characteristic electrical signal in the ground (see *Nature* 375, 617; 1995). Papadopoulos's claim was based on the view that an earlier earthquake in north-western Greece would trigger another rupture within two months. He claims to have been ignored, despite sending in "an accurate prediction" of the time, magnitude and location of the June earthquake, which took place in western Greece.

After a preliminary three-month investigation, the prosecutor has ordered Papanikolaou, who has already had to defend himself once, to testify for a second time. Kostas will then decide if he needs to stand trial on the grounds that heeding the warnings — regardless of their scientific credibility — would have saved lives.

Papanikolaou says he "cannot believe" the decision to charge him, particularly as he is one of a small minority of seismologists in Greece who believe VAN may work. More importantly, he claims to have taken preventive action partly on Varotsos's advice, which turned out to be inaccurate.

"After the May earthquake, I consulted several colleagues, including Varotsos, and decided to alert emergency services in the Thessalia area of central Greece to prepare for a possible earthquake in June," he says. "It is not my fault that the earthquake took place 100 km south," in the seaside resort of Egion. "This is so ironic, as I am the one accused by colleagues of risking my credibility by helping Varotsos to set up some of his monitoring stations."

The prosecutor's decision has stunned the scientific community. Thirty Earth scientists — including the directors of three major geophysics laboratories — have agreed to testify later this month in Papanikolaou's defence. "This business is a tragedy," says George Stavrakakis, acting director of the Geodynamic Institute of the National Observatory of Athens. "Dimitris

[Papanikolaou] cannot be held responsible when the whole seismological world believes that none of these methods is an acceptable method of earthquake prediction."

Varotsos claims that he has predicted the last three earthquakes in Greece using VAN. But seismologists remain sceptical, pointing out that the technique has not been properly peer-reviewed, and the physics behind the predictions remains unclear.

Both Papanikolaou and Stavrakakis are also angry at the public prosecutor's decision not to take evidence from senior colleagues during the investigation, apart from

IMAGE UNAMAGABLE FOR A CORNABGET FORESOUGHT REASONS

Greek tragedy: would listening to warnings of June's earthquake have saved lives?

Varotsos and Papadopoulos. "If he [the prosecutor] had listened to any one of us, he would have come to a different conclusion," says Stavrakakis.

The prosecutor's decision also puts Varotsos in a tight spot, as Papanikolaou was perhaps his closest supporter among seismologists. But, with both the public and scientific image of VAN at stake, Varotsos has no hesitation in declaring Kostas's decision as "a good initiative".

He says peer review "cannot go on forever" if lives are at risk, just as pharmaceutical companies are allowed to halt trials for AIDS drugs and rush them to market if patients show signs of living longer. "If anyone has doubts about VAN, they need to come out with concrete scientific facts. So far, I have predicted the last three big earthquakes in Greece, which, statistically, is a one-in-seven million chance of happening. This is no fluke, it is based on good science."

Later this month, Varotsos will meet the Greek Prime Minister, Andreas Papandreou, who is believed to have promised more funds for VAN research. The University of California at Berkeley will hold a three-day workshop on the subject on 10–12 October.

Ehsan Masood

Royal Society plans genetics meeting with actuarial profession

London. Britain's Royal Society is planning to link up with the actuarial profession to explore ways in which mathematicians and epidemiologists can help the insurance industry to assess the health prospects of individuals on the basis of knowledge of their genetic make-up.

In particular, the society is planning a meeting next summer on genetic screening and the insurance industry, to be organized jointly with the Institute of Actuaries. Among other topics, the meeting will discuss "how genetic analysis could be used to identify potential morbidity and mortality in groups and individuals".

The proposed meeting was described last week by Sir Brian Jenkins, master of the Worshipful Company of Information Technologists, at the launch of an initiative known as the City, Science and Technology Dialogue, which is intended to stimulate closer links between Britain's research and investment communities.

In his speech, which was sponsored by the Department of Trade and Industry, Jenkins said that the main purpose of the new initiative was to make city investors more aware of the impact of science, engineering and technology on the health of the economy. Its ultimate aim is "to reinforce the position of the City of London as a knowledgeable capital and securities market for science-based companies here and abroad".

Jenkins described the proposed joint meeting of the Royal Society and the actuarial profession — whose participants, he said, will include researchers from the fields of applied mathematics, epidemiology and demography — as the type of joint research endeavour between scientists and investors that might be promoted under the new initiative.

Officials at the Institute of Actuaries, which is still discussing the detailed programme of the proposed meeting, say that its purpose would be to make actuaries aware of the way in which modern genetic discoveries — many based on the unravelling of the information contained in the human genome — will inevitably affect the way in which health risks are assessed.

Sir Michael Atiyah, the president of the Royal Society, and one of Britain's foremost mathematicians, said that the topic of the use of genetic screening by the insurance industry offered an important opportunity for collaboration between the scientific and financial communities. "Actuaries form a very logical body to partner," he says.

Indeed, Atiyah points out that London's role as both a major international centre for the insurance industry and a focal point for Britain's internationally recognized skills ►

► in both genetics and mathematics means that it is well placed to play a leading role in linking the two. "The information is all there," he says. "There is no reason why we cannot put it all together."

At the same time, Royal Society officials say that they are well aware of the contentious nature of many of the issues raised by proposals to integrate genetic information into the provision of health and life insurance. They are therefore keen that discussions of the implications of genetic discoveries for the actuarial profession are balanced by consideration of the way in which the use of such information by insurance companies is regulated.

Roy Anderson, Linacre professor of zoology at the University of Oxford, and one of the organizers of the planned meeting, says that its aim will be to explain to the actuarial profession the scientific issues raised by modern genetics, and to provide precise information about "what is and what is not possible".

Anderson says the meeting is likely to address both the practical and ethical issues raised for actuaries, pointing out that the impact of genetics on the insurance industry are likely to be limited on the short-term, but over a longer time scale will become significant. "The Royal Society does not get involved in too many issues at the interface between science and society, but I am convinced that this is one area in which it should be active," he says.

In his speech last week, Jenkins said that the overall purpose of projects such as the genetic screening meeting was "to promote and present scientists and investors as natural partners".

David Dickson

Space station gets pledge of 5-year backing in Congress

Washington. In a bid to reassure the United States' international partners of its commitment to the space station, the House of Representatives has passed a bill that would authorize \$13 billion of funding for the project through to its completion in 2002. The multi-year authorization bill, which was sponsored by a bipartisan group led by Bob Walker (Republican, Pennsylvania), the chairman of the House science committee, passed the House on a voice vote last Thursday, with no dissent.

"Our partners overseas keep getting mixed messages," says Dan Goldin, the administrator of the National Aeronautical and Space Administration (NASA). "This will reassure them that America is serious about the space station programme". According to Walker, the House "has taken a giant leap forward in securing the trust of those nations that have entered into co-operative agreements with us."

The passage of the bill indicates the extent to which support for the space station has firmed up in the House of Representatives. In 1993, the project only survived there

by a 216-215 margin; but this July, an amendment to kill it fell overwhelmingly, by 287 votes to 132. Last week, opponents didn't even bother to call for a vote.

But the bill itself is primarily of symbolic importance. To become law, it needs to pass in the Senate. Even if it does, authorization

^{NASA} bills merely set funding ceilings; actual budgets are determined by annual appropriations bills.

Walker says that one key Senator — Conrad Burns (Republican, Montana), chairman of the science, technology and space subcommittee — will "aggressively pursue" a similar bill. But Senate staff say the upper house will be reluctant to change its normal practice of passing an annual authorization

bill for the whole of NASA.

Supporters believe, however, the bill's passage into law would place a moral obligation on Congress to complete the programme. It has been argued that the Superconducting Super Collider, cancelled by Congress in 1993, would have been more secure from attack if funding had been authorized for completion. **Colin Macilwain**

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Up and away? Promise 'will reassure' NASA's partners in the space station.

Europe edges towards agreement over space funding

Paris. With two weeks to go before the space ministers of member states of the European Space Agency (ESA) have to take some crucial decisions at a meeting in Toulouse, France, negotiations remain deadlocked about both Europe's contribution to the planned international space station and future funding levels of European space science missions.

But although large obstacles to agreement remain, European space officials say that considerable progress has been made over the past few weeks. In particular, Germany and France seem closer to an agreement about their participation in ESA's planned contribution to the space station.

France has already withdrawn its proposal to substitute a French-built crew rescue vehicle (CRV) for the planned space laboratory, which would be built by Germany and Italy (see *Nature* 377, 191; 1995). Furthermore, during recent interministerial meetings, France has said it would agree to raise its funding for the station over the period 1996-2000 to around ECU400 million (US\$488 million).

In return, Germany, the ESA member state with the greatest interest in the space station, is said to be ready to invest in the French-led programme to upgrade the Ariane V launcher, and to agree that ESA's contribution to the station should include money for further technical studies of France's CRV project. Germany has already agreed to pay ECU600 million towards the station programme.

The joker in the pack remains Italy. Earlier this month, the Italian Space Agency (ASI) informally told ESA that it would be unable to pay its expected cash contribution of ECU300 million to the station. Instead, it offered around ECU100 million, with the rest being paid in kind.

This proposition might have ended ESA's hopes of being able to pay for its planned ECU1.4-billion contribution to the station. But the emerging consensus between France and Germany is putting pressure on Italy not to break ranks, and the Italian government is now said to be considering raising ASI's budget in order to allow it to increase its participation.

Meanwhile, at a meeting last week of the ESA council, made up of representatives from each of the agency's member states, Britain refused to budge on its demand that ESA reduce its budget for space science by 25 per cent over five years. But Ian Corbett, the director of science at the UK Particle Physics and Astronomy Council, and the UK's representative on the ESA council, says that "we and ESA recognize that a solution must be found; we live in a real world".

Indeed, in a partial concession to the British demands for reduced funding of the space science programme, ESA has offered to freeze 2.5, 5, and 7.5 per cent of the programme's budget in 1996, 1997 and 1998 respectively. The sum frozen would then be released only after a unanimous vote of the ESA council.

While the United Kingdom is holding out for a better offer, Germany is said to be considering whether to accept this proposal. Both have opposed a proposed increase in the agency's science budget in line with inflation, Germany suggesting it should be kept constant in cash terms. **Declan Butler**

US laboratories escape a radical overhaul...

San Francisco. President Bill Clinton has told the US Department of Energy (DoE) to keep the Lawrence Livermore, Los Alamos and Sandia National Laboratories intact, rejecting previous recommendations that had included privatizing all three laboratories, consolidating key programmes and shifting all weapons design to Los Alamos.

Hazel O'Leary, the Secretary of Energy, said last week that the administration had compared the savings of consolidating the programmes against the cost of removing one weapons laboratory from the so-called 'stewardship' programme for the nation's

nuclear weapons stockpile. Officials had concluded that the savings would be insignificant compared with the harm over time to public confidence in the stockpile.

O'Leary added she had also been swayed by the diversity of Lawrence Livermore's research programmes, which include environmental technology, laser technology and the human genome, in addition to its national defence work.

But the announcement has not given laboratory officials freedom to continue as previously. The three laboratories were ordered to review their management infra-

structure for unnecessary costs, to sharpen their mission focus and to reduce duplication, as well as to eliminate lower priority programmes. Observers said the ability of the laboratories to shake off the grip of DoE bureaucracy will be critical to their cost-saving efforts.

C. Bruce Tarter, the director of the Lawrence Livermore Laboratory, says a cost-cutting task force of technical and administrative staff will make their recommendations later this month. The committee has been asked to find ways to cut between \$25 and \$75 million from a \$875-million budget. "It's easy to cut costs; what's hard is to cut costs and still preserve the character of the place we're in," says Tarter.

A federal-level laboratory management board set up earlier this year will provide its own recommendations for the laboratory. M. R. C. Greenwood, a former White House science official who is a member of the board, says it is important to maintain the scientific and technical capacities of all three laboratories. In general, she says, the board aims to streamline the laboratories in much the way as private industry has improved its operations, by reducing levels of bureaucracy, clarifying reporting lines and moving decision-making to a level at which research workers are closer to the issues involved.

Tarter said it is too early to tell which programmes may come under fire at Livermore, or how many additional positions will be cut. Over the past two years, Livermore has cut its workforce by 1,500, primarily from its full-time staff and contract labour.

But Tarter adds that civilian programmes such as studies on magnetic fusion, climate change, the human genome and the environment, may be forced to take cuts. In contrast, continuing research on nuclear weapons, non-proliferation, arms control and cleaning up nuclear waste in the environment are expected to do relatively well in the forthcoming budget. Laser-isotope technology also remains well-funded under a contract from the public/private US Enrichment Corporation.

Sources in Congress say that, in general, basic research programmes are likely to have a better chance of survival than applied work, as legislators believe that too many applied programmes carry out work that should be pursued by private companies.

There will also be competition for new projects. Livermore, for example, is competing with Lawrence Berkeley Laboratory to run the National Energy Research Supercomputer Center, a \$45-million programme that the Energy Department aims to reduce by about 20 per cent. The centre, which has been housed at Livermore for 21 years, is responsible for three supercomputers and employs 110 people.

Sally Lehrman

... as UK backs down on reform ideas

London. In a humiliating rebuff to its own efforts to increase the efficiency with which publicly funded research laboratories are managed, the British government has rejected all three proposals put forward last year by a four-member 'scrutiny' team following visits to 53 research institutions.

Ian Lang, the president of the Board of Trade — and since July the cabinet minister responsible for science — announced last week that the government will not proceed with the team's controversial suggestions that the laboratories should be grouped together, either by discipline or by geographical location.

Nor will it seek to ensure changes in the way in which the laboratories, belonging in particular to the Biotechnology and Biological Sciences Research Council (BBSRC) and the National Environment Research Council (NERC), are run by appointing new 'directors of rationalization' to oversee their reorganization.

Lang did announce that the government is to undertake yet further reviews of all public research establishments, including those run by the research councils that were not covered by last year's investigation, which he promised "will be rigorous in their examination of the options for privatization".

But many see this as little more than an attempt to disguise the government's failure to produce a substantial case for extending its privatization plans to all sectors of the publicly funded scientific community.

The initial proposals to reorganize the research laboratories came under heavy fire from almost all parts of the community. The Royal Society, for example, published a swingeing attack on the scrutiny team's proposals last November, describing the plans as a basic misunderstanding of the role of the laboratories (see *Nature* 372, 122; 1994).

In a statement issued as a formal response to a report from the House of Commons select committee on science and

technology, Lang said that the government still intends to keep the pressure on research councils to adopt a more market-oriented approach to the way their managers operate in order to increase the effectiveness of public funds.

Even this, however, is likely to come under increasing attack as the opposition Labour party, currently enjoying a substantial lead in public opinion polls, targets the government's privatization record, both in science and elsewhere, in the run-up to the next general election.

Last week, for example, in a discussion document aimed at influencing the Labour party's thinking on science, a group known as the SET Forum, convened by Hilary Rose of the University of Bradford and Steven Rose of the Open University, claimed that the government's efforts to restructure public research laboratories, rather than creating more effectively managed institutions, had merely led to "shrinkage and demoralization".

The two authors, echoing suggestions previously endorsed by labour unions representing scientists and other bodies, suggest that the Labour party should halt any further dismantling of the public SET sector pending a review "not focused on marketability but on function and need". In a pamphlet entitled *Shaping the Future*, they write that "the heart of our criticism of the Conservatives today is not that they do not know what needs to be done, but that their ideological bias still makes it impossible for them to do it".

So far, the Labour party itself, which last week launched a national consultation exercise with industry, the academic community and the public before drawing up its formal science policy proposals, has made no formal statement on its plans for the public research laboratories. When it does so, however, proposals for 'networking' such laboratories along the lines indicated by Rose and Rose are expected to appear high on the agenda.

David Dickson

Japan opens new era in university funding

Tokyo. A sweeping reform of the way in which the Japanese government funds research, particularly in universities, has been launched with the introduction of a new grant scheme by an agency affiliated with the Ministry of International Trade and Industry (MITI).

The first grants from MITI's New Energy and Industrial Technology Development Organization (NEDO) were announced last week, with 95 of 109 going to universities. This marks the first time that a government department other than the Ministry of Education, Science, Sports and Culture (Monbusho) has been allowed to award a substantial number of large grants to university researchers (see *Nature* 376, 110; 1995).

NEDO has won further funds for the scheme, which is open to researchers in universities and national research institutes, in a supplementary budget announced on 20 September, and MITI has moved to institutionalize the scheme in its budget request for next fiscal year (which begins on 1 April 1996). Furthermore, both Monbusho and the Science and Technology Agency (STA), formerly bitter rivals, have announced similar schemes to fund research within each other's domains.

Taken together, the new schemes are the first examples of multiple-source funding across the spectrum of government research; previously, each ministry and agency usually only funded research organizations within their sphere of control. Competition for the first batch of grants, each worth ¥50–200 million (US\$0.5–\$2.0 million), was fierce; applicants outnumbered recipients by a factor of 20. The grants provide a "very

good opportunity for national institutes and university researchers to compete and survive", says one successful applicant, Yoji Mitsui of MITI's National Institute of Bioscience and Human Technology.

The grant approval process involved an assessment by a committee drawn from universities, national institutes and the private sector. The grants are intended to lay the foundations for new industrial technologies in fields such as new materials and biotechnology. Predictably, most of the grants have been awarded to leading national universities, and two-thirds have gone to laboratories in the region centred on Tokyo.

The new funds are expected to be widely welcomed in universities because of the low level of Monbusho's grants. The education ministry's budget for competitive grants for this fiscal year totalled only ¥92.4 billion, most of which is parcelled out in small grants of only a few million yen. The new NEDO awards, totalling ¥10 billion, are therefore a significant boost to university research. Furthermore, each NEDO grant includes money for one or two postdoctoral fellowships, a factor that is missing from most existing Monbusho grants.

The NEDO funds come out of a special supplementary budget prepared earlier this year to cope with the recent earthquake disaster in Kobe. NEDO has won a further ¥5 billion for the scheme in the most recent supplementary budget, and MITI has applied for ¥2.65 billion in next year's budget for a similar scheme that will run on a regular annual basis.

Until now, Monbusho has been reluctant to allow other ministries and agencies to

provide funds to Japan's universities, over which it has direct control. But a spokesman for the ministry says that it is happy to cooperate with the NEDO scheme, and would have no objection to grants continuing on a regular basis, or to other agencies deciding to award funds to university researchers.

In a remarkable about-turn on previous policy, he says, MITI and Monbusho have "transcended the idea of individual territories" in order to develop Japanese science and technology in the best possible way.

In line with this new mood of cooperation, the STA has announced it will vastly expand its support for university research. Starting with ¥5.1 billion requested in 1995's second supplementary budget, the STA will offer 48 grants, each worth ¥1 billion over five years, to applicants from universities, as well as national research institutes operated by MITI and the STA itself.

This programme will be further expanded with another ¥15 billion requested by the STA for fiscal 1996. This money, which will be part of STA's ordinary annual budget, will be used to fund a further 40 projects.

Decisions on who will receive these grants will be made by a committee formed by the STA, drawing on researchers in universities, national institutes and the private sector. Applications for the first batch of grants are due in November, and successful candidates will be advised in January.

Perhaps the most surprising development of all comes from Monbusho itself. The ministry has asked the government for ¥11 billion in the next fiscal year to provide large grants not only to university researchers but also to researchers in national institutes overseen by MITI and STA.

Akito Arima, former president of Tokyo University and current president of the Institute of Physical and Chemical Research (RIKEN), who has been campaigning for the reform of Japan's university research system, says the new spirit of cooperation between different government agencies is "extremely good" for research in universities and national research institutes, as it opens up multiple sources of funding. But he warns that it may "only stimulate applied science", as disciplines which lack immediate applications, such as astronomy and high-energy physics, will not be covered by the new schemes.

Fumio Kodama of Tokyo University's Research Center for Advanced Science and Technology points out, however, that it would be strange for either MITI or the STA to fund academic science, as these institutions are dedicated to supporting industry-related research. Rather, he suggests that Monbusho should play a "balancing role" by channelling more of its money into purely academic research.

Stephen Barker

Microchip Mark 1 goes up for auction

London. A section of computing history was due to go under the hammer this week with the sale of part of Charles Babbage's Difference Engine No.1 (right) at the London auctioneers Christie's.

The device is one of six assembled in 1879 from the remaining components of the original Engine by Babbage's son Henry.

The section offered for sale, owned by the family of a grandson in New Zealand to which it had been sent, was expected to fetch £50,000. Babbage was unable to finish the machine, although the Science Museum in London has managed to construct a working model of a revised version of the Engine, using the original designs.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Babbage, a one-time Lucasian professor of mathematics at the University of Cambridge, became interested in mechanical calculation as a way of solving errors made by the human brain.

E. M.

Christie's, South Kensington

Medical lobby seeks cuts in US health research agency

Washington. Uncertainty is hanging over the future of a US health research agency responsible for improving the quality and efficiency of health care in the United States, as Republicans in both houses of Congress seek major cuts in its budget.

Officials at the Agency for Health Care Policy and Research (AHCPR), best known for its development of clinical practice guidelines, fear that delays in approving the health-funding bill in the Senate will give a little-known doctors' group, offended by the agency's conclusions about excessive surgery and other issues, more time to persuade senators to inflict deep cuts on the agency.

The House of Representatives has already passed the bill, which funds the Departments of Labor, Health and Human Services, and Education, with an amendment by Sam Johnson (Republican, Texas) providing just \$66 million for the agency in the fiscal year which officially started this week — less than half its current \$159 million annual budget.

The Senate bill, currently deadlocked over cuts in social programmes (see *Nature* 377, 187; 1995), would give NIH an overall increase of 2.7 per cent over this year's level. The AHCPR would have its funds cut to just \$127 million. "High quality, comprehensive research cannot absorb a reduction of this magnitude and still yield valid scientific results," says Clifton Gaus, administrator of the agency, which has already cut its non-university research grants by 35 per cent.

The AHCPR carries out research aimed at improving the quality of health care, with an emphasis on women, minorities and rural health. "There are going to be economic winners and losers when you do this type of research," says Robert Griffin, a spokesman for the agency. Two years ago, for example, cataract surgeons were angered by the agency's conclusion that surgery was not always the best treatment for cataracts.

The winners include advocates of those infected with HIV, who have referred to

AHCPR's data when defending AIDS service programmes from cuts. The AIDS Action Council, for example, which promotes AIDS research and services, plans to arm itself with research supported by AHCPR and published last month that shows no relation between injection-drug use and the progression of HIV.

The AHCPR's recent troubles began after it released practice guidelines last year on lower-back pain, claiming surgery was unnecessary in most cases in which the pain lasts less than three months. Neil Kahanovitz, a Virginia spinal surgeon affiliated to the North American Spine Association, a group of spine surgeons, subsequently formed a group to lobby Johnson and other House legislators to persuade them to support a cut in the agency's funding.

"It's not the content of the guidelines he dislikes, it's that so many other agencies are doing the same work," Dayna Cade, spokeswoman for the lobbying group said. But Cade's defence rings hollow, as no other government agency has a mission similar to that of AHCPR, formed in 1989 to improve the quality and cost-effectiveness of care.

Most medical groups, including the American Academy of Orthopedic Surgeons, have supported AHCPR's work. But Johnson sides with the Center for Patient Advocacy, in which a number of spine surgeons from Texas are active. "We don't need, nor can we afford, the AHCPR," Johnson said when arguing for his amendment. An aide explains that Johnson has proposed cuts to the agency's budget because of his own concerns and those of health providers, including Kahanovitz.

The AHCPR has now embarked on its own promotional effort. "Just as the NIH is shaping the future of medicine by mapping human genes, AHCPR is providing the road map to guide the public and private sector in their quest to lower costs without hurting the quality of health care," says Gaus.

Adrienne Appel

OECD extends 'megascience' clearing house

Paris. The so-called 'megascience' forum, a body set up in 1992 by the Organization for Economic Cooperation and Development (OECD) as a clearing house for information on big science projects, has had its mandate renewed for a further three years.

The decision was taken at a meeting in Paris last week of the OECD's high-level Committee for Scientific and Technological Policy. The forum has produced reviews of large projects in astronomy, deep Earth drilling, global change, oceanography, and neutron and synchrotron radiation sources.

The meeting also agreed that the forum should be given responsibility for monitoring and catalysing international scientific collaboration. In particular, it will be allowed to set up working groups to explore new areas for cooperation, made up of government officials and scientists.

Peter Tindemans, the chairman of the forum, says the new arrangement will give it "greater political clout", and extend its remit beyond big science projects to include cooperation in general — for example, in environmental research.

Declan Butler

Science stars fail to shine in challenge from student team

London. Britain may need to re-evaluate its concept of scientific literacy following an embarrassing defeat by a team of students of a Royal Society panel led by Lewis Wolpert, professor of biology at University College, London, and chairman of its committee on the public understanding of science, in a mock round of the television quiz show 'University Challenge'.

The Royal Society team also included the government's chief scientific adviser, Robert May, and Sir Martin Rees, Astronomer Royal, who is this year's president of the British Association for the Advancement of Science. It had challenged the current University Challenge champions, Trinity College, Cambridge, to a general knowledge quiz chaired by the show's regular host, Jeremy Paxman. The quiz was held last week in the society's newly refurbished Wellcome Trust lecture hall.

In addition to being comprehensively beaten — with a final score of 210 points to 120 — the Royal Society fellows appeared to suffer an attack of collective amnesia when attempting to answer questions not merely on science, geography and current affairs but also on the history of the society itself.

Rees later put the defeat down to "slow reaction times, and slow recall from our store of knowledge". But even this failed to explain why, during the part of the quiz when team members are allowed to confer before giving an answer, the Royal Society fellows were unable to reply even to straightforward questions from their own areas of education and experience.

Wolpert, who introduced himself as coming from South Africa, failed to recognize a prominent Pretoria monument. And May, who hails from Australia, needed reminding that Perth is the only Australian state capital not to be named after a person.

Despite much support from members of the audience, who whispered answers loudly from the floor and burst into applause whenever the home team answered correctly, the team was unable to name either where the society's first meeting took place, or the device — a magnetron — that generates microwaves in a microwave oven.

At the end of the contest, Paxman remarked that the result showed that C. P. Snow's concept of science and the arts as representing 'two cultures' appeared to be alive and well.

Others suggested that the Royal Society's fellows had not been particularly looking forward to the contest. When news of the impending challenge became known, they "did not exactly queue up to be part of the team", according to one observer.

Ehsan Masood

Embattled US ecologists

SIR — The embattled ecologists of the United States are faced with a multitude of obstacles and choices, including budget cuts and demonization from the Right. However, one crossroads that they thankfully are not at is the "level-headed compromise" that is characteristic of political animals, rather than the ecologically concerned (*Nature* 376, 461; 1995).

The implication throughout Henry Gee's leading article is that US ecologists are to blame for their public image 'problems' which have resulted in, among other events, the failure of the United States to ratify the Biodiversity Treaty from the Rio de Janeiro conference. This is supposedly because they have "failed to distance themselves from unfocused environmental activism".

At some point scientists are faced with politico-cultural realities associated with their subject of study which is often also their passion. In his waning years, Einstein advocated elimination of nuclear weapons manufacture, the development of which was largely facilitated by his own discoveries. Ecologists today are faced with an impending disaster not of their own making that may nevertheless destroy the object of their own focus. Environmental legislation in the United States is fraught with the very compromises Gee advocates. But the inevitable result of continuous compromise is an end product that has no discernible relationship to the original article.

As Gee points out, US ecologists should indeed look to the United Kingdom for lessons on ecological projection and protection. This cultural ancestor of the United States has managed to compromise its ecology to the point where it can boast no wilderness areas, ocean-to-ocean pasturage and urban development, and a regularly declining economic situation. Without avid advocacy, US ecologists of future centuries may also find themselves studying hedgerow 'ecosystems' in the formerly wild Rocky Mountains.

Contrary to Gee's assertion, US ecologists cannot compromise on end-game legislation such as the Endangered Species Act, now under review by Congress under the aegis of their industrial suppliers. The act comprises a stop-gap measure to reduce anthropogenic extinction of non-human species and has proved functional, an illustrious example being the national bird of the United States, the bald eagle. The role of publicly funded ecologists in the United States is to inform the public and/or the voters of the goings-on in the natural world. If human societies and agencies cause damage to our world, then ecologists should welcome even the odd "hirsute" environmental activist as a

bedfellow in their mission to expose this damage. As scientists we can be both objective observers and passionate advocates of the natural processes we love. Indeed, we have been doing so for centuries already.

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Katyn massacre

SIR — I read with interest your leading article on rewriting history (*Nature* 376, 539, 1995) and I agree with your view that teaching contemporary history is important. In this spirit, I should like to elaborate on your reference to the massacre at Katyn.

In 1939, 17 days after Germany invaded Poland from the west, Soviet forces invaded the eastern half of Poland without declaring war. Among other people detained and transported inside the Soviet Union were more than 20,000 Polish officers. They were kept in several different prison camps, the three best-known being Katyn, Starobielsk and Ostaszkowo. Virtually all were exterminated in the spring of 1940, probably by the order of Stalin himself. Executions were carried out in various localities close to the camps. The mass grave near Katyn was the first one to be discovered by the Germans after they in turn invaded this part of the Soviet Union in 1941. It contained more than 4,000 bodies.

Nobody knows all of Stalin's motives and I agree that vindictiveness played a large role. However, it seems that executions of Polish officers were something more than an act of vengeance. Most of those officers were not professional soldiers, but reservists mobilized at the beginning of the German invasion of Poland.

There were many thousands of other soldiers and civilians in the Soviet prison and labour camps during the Second World War. Many of them perished, but as far as I know they were not subjected to mass execution.

Extermination of the officers was probably a part of the policy to annihilate the Polish intelligentsia, which was carried out by both the German and Soviet occupying forces during the Second World War, and continued for some time after the war by the communist government installed by the Soviets in Poland.

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Visual processing

SIR — The results of Morrone *et al.*¹ imply the existence of specialized detectors that integrate signals of different directions from different locations, leading to the inference that composition in (visual) art is a consequence of the existence of specific circuits.

Composition² means the body of rules defining the disposition of the parts of a work of art to draw the observer's attention to the essential. In a sense, the viewer perceives (mostly unconsciously) a 'super-pattern', a 'compositional *gestalt*' in Köhler's sense³. Among the dominant compositional elements are the two diagonals, the circle, some straight horizontals and verticals as well as their combinations. What is remarkable about the rules of composition is their generality. They are consistently respected from 30,000 BP (Chauvet Cave⁴) to the twentieth-century abstractions and over the five continents, so that they seem to be universal and not merely artistic conventions. So why not conjecture that composition in visual art is determined by innate radial, circular and translational visual processing?

Michel Benarie

European Cultural Heritage Newsletter,
20 Bd Jean Pain,
38000 Grenoble, France

1. Morrone, M. C. *et al.* *Nature* 376, 507–509 (1995).
2. Poore, H. R. *Composition in Art* (Avenel, New York, 1967).
3. Köhler, W. *Gestalt Psychology* (New York and London, 1929).
4. Chauvet, J.-M. *et al.* *La grotte Chauvet* (Seuil, Paris, 1995).

Unsung hero

SIR — Marconi's contribution to wireless transmission is often mentioned in scientific journals (see, for example, H. C. Alexander, *Nature* 373, 184; 1995). Sir Jagadish Chandra Bose (no relation to Satyendranath Bose of Bose–Einstein statistics fame) made his contribution to wireless transmission before Marconi.

Some years ago, in *Physics Today* (39, 36–44; 1986), Dr W. A. Blanpied wrote:

In 1895 Bose gave a public lecture at Town Hall, Calcutta, in which he demonstrated for the first time wireless transmission of electromagnetic signals through solid walls; it quickly made him famous throughout Bengal. As a result of the growing fame, the government of Bengal sent him on a nine-month lecture tour of Europe, where in December 1896 he repeated his demonstrations of wireless transmission at the Royal Institution before an audience that included Lord Kelvin. That event anticipated by a year Guglielmo Marconi's more celebrated (and commercially exploited) wireless-transmission demonstrations in the same city.

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When to help farm animals grow faster

The European Commission has broken with precedent by sponsoring an independent scientific conference on the role of growth-promoting substances in animal husbandry.

WHEN athletes use anabolic steroids to enhance their performance on track and field and are found out, they suffer dire penalties. Often, they are banned from further competition, either for some years or even for the rest of time. Olympic performers tearfully on the way home after being stripped of medals they supposed they had won are a common sight. The principle underlying these draconian measures is simple; athletic competitions are supposed to be tests of natural athleticism, whose artificial enhancement by means of drugs is manifestly unfair. There is naturally less concern with the damage athletes may do to themselves by the long-term use of drugs.

But there are other fields in which drugs that improve performance are nevertheless widely and legally used. Animal husbandry is the outstanding example. The various steroids that human weight-lifters may use to improve their competitiveness will also enable beef cattle to accumulate muscle tissue more quickly than would otherwise be the case. The result is beef in which the ratio of fat to lean meat is enviably low, and which, to the profit of enterprising farmers, can reach the market relatively quickly. The use of steroids and other classes of drugs to enhance the growth of commercially domesticated animals is now widespread, but not in Western Europe; since 1989, the use of growth-promoting substances in livestock feed has been prohibited by a series of directives by the European Union (EU).

The origins of the EU ban are complex. Environmental pressure groups were among those who proclaimed that the residues of growth-enhancing substances in meat offered for consumption would, over the years, damage the health of those who ate it. And the effects are not always simply toxicological. Who can say whether traces of antibiotics in meat do not encourage the emergence of resistant microorganisms, thus disadvantaging even vegetarians? There is also concern that growth-promoters will damage the health of animals treated with them. In the late 1980s, the European Parliament was a powerful sounding-board for these anxieties. Farmers' organizations and EU officials might well have been more vigorous in their opposition to the ban if it had not been for Europe's surplus production of beef.

Not all the consequences of the European ban have been beneficial. For one thing, meat producers outside Europe have protested that Europe's ban on imported

meat produced with the help of growth-enhancing chemicals is a "non-tariff restraint on trade". Not surprisingly, the ban has also strengthened the pre-existing illicit European trade in growth-enhancing chemicals. From time to time, farmers and others are prosecuted for feeding these materials to livestock. Because the initiative for prosecution lies with national inspectors, nobody knows how general is evasion; there are fears that the illicit market may be supplied with cocktails of growth-enhancers made up by unregulated cowboy manufacturers, with dangers comparable to those all-too-apparent in the illicit trade in what are laughably called 'recreational' drugs.

That is the background to an unusual scientific conference to be sponsored from 29 November to 1 December by the agriculture directorate of the European Commission, and of which I will be the chairman. The intention is to review the scientific evidence bearing on the use of growth-promoting substances in animal husbandry, and to make an assessment of the present state of knowledge. To ensure that it cannot afterwards be accused of being partial, the commission has delegated organization of the conference entirely to a small steering committee, whose members (apart from myself) are Professor François André (Laboratoire des Dosages Hormonaux, Nantes); Professor Carlos van Peteghem (Food Analysis Laboratory, Ghent); and Dr Francis W. Kenny (Central Meat Control Laboratory, Dublin).

The programme of the conference is built around three topics, each of which will be introduced by a review paper by a person who will then become the chairman of a working group of the issue. Thus Professor James Roche (Dublin) will deal with the variety of growth promoters now in use or on the horizon (which, of course, includes the transplantation of growth-promoting genes); Mr Jan Rud Andersen (Danish Meat Research Institute) with the analytical methods of detection; and Professor Fritz Ungemach (Institut für Pharmakologie, University of Leipzig) with the health risks of growth-promoters.

What follows is an account of the ways in which the conference is unusual ... and an appeal for help. First, the commission's delegation of responsibility to the steering committee has been complete; there have been occasions when the commission's officials have been asked to let us pursue our discussions on our own. The 60 or so par-

ticipants are being invited on our recommendation, on the basis of what we know of their scientific contributions in the field; they come not only from Europe, but from other major meat producers such as Australia, Canada and New Zealand as well as the United States.

After some heart-searching, however, we have decided not to invite as participants scientists affiliated with the major veterinary drug manufacturers or other interested parties, farmers' organizations for example. That concession to political correctness does not imply that such people are not good scientists, or are unable to make unbiased statements, but reflects our wish that the conclusions of the conference should not afterwards be attacked for representing industrial opinion.

Such people are, however, invited to bring to the attention of the conference data and other material gathered in the course of their scientific work by sending it by e-mail to conf95@mhsg.cec.be. Data or general comments, if received before 10 November, will be put through a process of peer review. Comments and data received after that date will be acknowledged and read by the steering committee, but will necessarily be given more cursory attention.

In addition to the regular invited participants, the commission also plans to invite a number of interested parties in Europe. Chief among them are the members of the European Parliament who have taken a continuing interest in growth-promoters, as well as consumers' organizations in Europe and elsewhere. They will be allowed to listen to the proceedings, to make statements of predetermined length if they can persuade the chairman of the session concerned that they have something apposite to say and, finally, to take part in a half-day debate on the afternoon of 1 December when the general conclusions of the conference will be presented orally.

Undoubtedly, it will be discovered that there is much still to be learned about the risks of using growth-promoting substances in animal husbandry; one goal is to identify specific areas in which more understanding is needed. On the face of things, there is an urgent need for a better understanding of the effects of trace residues on human physiology. But the ideal will be that the conference should inform the continuing dialogue between the commission and the European Parliament with a substantial basis of understanding.

John Maddox

A supernova engine turns over

Eric S. Myra

WHAT forces are at work when a massive star collapses and explodes? A precise answer to this question has remained elusive despite the persistence of more than a generation of astrophysicists. Recent research suggests, however, that this long-standing mystery may finally be solved. In the *Astrophysical Journal*¹, Adam Burrows, John Hayes and Bruce Fryxell provide the most convincing evidence so far that neutrino-driven convection powers the explosion of a type II supernova.

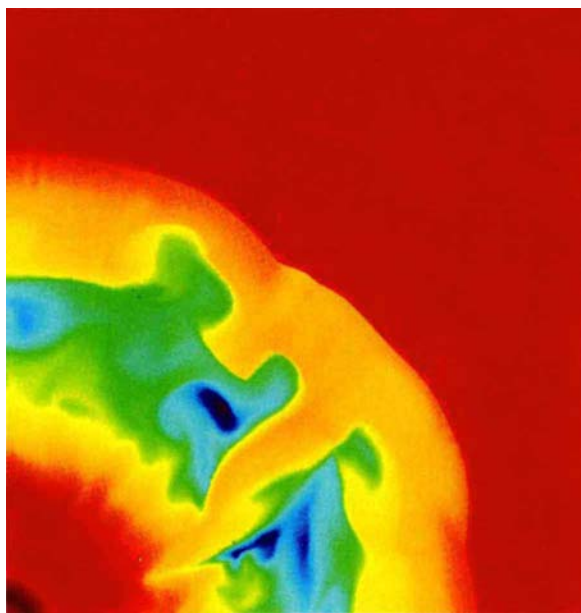
The Universe's factory for heavy elements is the interior of stars larger than about 10 solar masses. An explosive mechanism, such as a supernova, is needed to liberate these heavy elements to produce the abundances that make up the interstellar medium and indeed our own bodies. The problem facing astrophysicists, however, is in describing how the material works itself free of the star. A supernova produces many times the energy required to get the job done, but the best theoretical models have trouble getting enough energy to the right place at the right time. The problem is thus one of thermodynamics — delivering the energy released in a collapsing star to the mechanical 'engine' that drives the explosion.

In the standard picture for type II supernovae, the progenitor star, whose life has been governed by nuclear fusion, evolves to produce an iron core of between 1 and 2 solar masses. As iron is the most stably bound nucleus, fusion cannot release further energy. The stellar core thus falls victim to other processes that drive its subsequent evolution, the most important being weak charged currents in the form of inverse beta decay. At densities that exist in the core (more than 10^9 g cm⁻³), protons start to absorb electrons, producing neutrons and neutrinos. Because electrons have been providing the dominant component of pressure, their disappearance leads to the core's rapid collapse in less than one second. The weakly interacting neutrinos initially stream from the core, but start diffusing and equilibrating with the matter as the collapse proceeds to higher

densities. It is the degree of this interaction with matter that is important later in the scheme of events.

Collapse would continue indefinitely were it not for the strong nuclear force. When the core approaches nuclear density (around 3×10^{14} g cm⁻³), the strong force arrests and reverses the collapse. (Nuclei are almost incompressible.) The sound wave from this 'core bounce' propagates out through the core to where the velocity of the infalling material matches the local speed of sound. Near this sonic point, a shock wave forms, which soon starts propagating outwards.

What happens next is the matter of controversy. It is agreed that the shock wave must somehow propagate to the edge of the core and eventually disrupt the overlying star. It is also agreed that a portion of the core remains behind to form a dense neutron star. What is controversial is how the shock wave is powered. Over the years, a number of mechanisms have been suggested, ranging (in broad terms) from a purely hydrodynamic shock² to a neutrino-diffusion-driven shock³.



The supernova shows one of its many multi-dimensional aspects in this plot of entropy distribution adapted by Burrows *et al.*¹. A wedge of the inner star is shown about 5 milliseconds before the onset of the explosion. Colours towards the red end of the spectrum are regions of low entropy. The shock wave is defined by the boundary between the yellow and outer red regions. The yellow to blue regions are at high entropy and occur where the shock wave generated by the core bounce has heated material through which it has passed. The marked departure from spherical symmetry in this region is caused by neutrino-driven convective instabilities. Of special note is a plume of descending material, making about a 30° angle with the horizontal. The hotter blue bubbles, of the sort seen near the plume, will shortly drive the explosion.

The problem with these mechanisms is that they have not lived up to their initial promise. Follow-up calculations that model the physics more completely rarely result in an explosion. What they show is a shock wave receding towards the centre of the core, weakened by nuclear dissociation and neutrino production in the accreted shock-heated material⁴⁻⁸. Such calculations generally assume that the star is spherically symmetric and hence are performed in one dimension.

That convection might supply the shock wave with energy is not a new idea and has had several incarnations over the years. Its most recent and persuasive champion has been Hans Bethe⁹, who argues that convective instabilities occur in the wake of the shock. These instabilities are caused by neutrinos, which interact in such a way as to heat the material directly behind the shock while cooling material that lies farther below. The resulting convective overturn transports energy, a portion of which is available to do work on the shock.

In an appealing argument, Herant and co-workers¹⁰ have extended these ideas to model the convective core as a vast thermodynamic engine. Through neutrino heating and large-scale convection, a cycle is created by which heat released in gravitational collapse is converted to hydrodynamic energy that powers the shock wave through the star. Given that convection is inherently a multi-dimensional phenomenon, Herant and colleagues test this idea with two-dimensional simulations and find conditions that yield vigorous explosions.

The new calculations of Burrows *et al.*¹ add weight to this idea. Although their analysis of events takes issue with the heat engine interpretation, they confirm the overall success of the convective mechanism. Their calculations employ what is, so far, the best combined treatment of two-dimensional hydrodynamics, convection, the equation of state and neutrino transport.

With this wealth of physical input, the multi-dimensional character of the supernova becomes compelling. For the explosion mechanism itself, the breaking of spherical symmetry emerges as a critical element of shock propagation. As the explosion proceeds, the persistence of this asymmetry can be further applied to the explosive production of nuclei, to the character of supernova remnants, and even to the observed dynamics of the remaining neutron star.

If large-scale convection truly occurs as these multi-dimensional models suggest, it is clear why one-dimensional convecting models have had so much trouble generating an explosion⁸. Such approximations for convection, by their very nature, have difficulty modelling multi-dimensional convective flow. Instead, they undergo a high degree of artificially prescribed mix-

ing of the heat engine's 'reservoirs'. Such a prescription is similar to modelling the peak efficiency of a refrigerator by leaving its door ajar.

Nevertheless, it is not possible to dismiss one-dimensional results completely. If convection is really the agent that powers the supernova, its success depends critically on the efficiency of the convective process^{1,11}. Because, in large measure, neutrino interactions determine this efficiency, the accuracy of a model's neutrino transport is paramount. Even with the advances that have been made by Burrows and co-workers, two-dimensional treatments of transport are still crude versions of the meticulous detail found in the best one-dimensional codes^{5,8}. Extending accurate transport to multiple dimensions is thus the next great challenge. The computing resources required to run such calculations promise to tax even the forthcoming generation of high-performance computers.

What results should be expected from future models? Clearly, the explosions obtained in the simulations carried out by Burrows *et al.* and Herant *et al.* inspire optimism that convection is the answer. On the other hand, intuition warns us that any process relying on neutrino-matter interaction will be hard to model and inefficient at best.

While awaiting further results, perhaps we should reflect on the years of parallel advances in nuclear physics, weak interaction physics, numerical models and computer architectures. Although such developments have not yet revealed a definitive supernova explosion mechanism, they have enriched our appreciation of the subtle interplay of many diverse branches of physics. If, in the end, it is concluded that neutrino-driven convection powers the supernova, it will be fitting that a particle as elusive as the neutrino was hiding the answer for so long. □

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Climate and the conveyor

Gerard C. Bond

ONE of the challenges facing palaeoclimatologists is explaining the sudden climate jumps that occurred every 1,000 years or so during the last glacial period. Many think that the solution to this climate puzzle will be found in the operation of the Atlantic Ocean's 'conveyor belt', and three recent studies — including one published this month in *Paleoceanography* — bear this out¹⁻³.

The Atlantic conveyor is part of the ocean's circulation which today carries warm surface water northwards and cold, dense deep water southwards. The processes that drive the conveyor are delicately balanced. Even a small change in the balance could alter its mode of operation and give the Earth's climate a serious jolt. This is amply demonstrated by the fact that today the enormous amount of heat released to the atmosphere as the conveyor belt's surface water cools and convects downwards keeps Europe's climate about 3 °C warmer than land at the equivalent latitude bordering the Pacific Ocean. If the conveyor were to switch off, Europe would suddenly become much colder. Because the conveyor is part of the global ocean circulation, the climate shift would probably be felt well beyond the North Atlantic. Computer models of ocean circulation have shown how easily the operation of the conveyor might be altered. Demonstrating that such changes actually occurred, though, has been difficult and controversial.

The three new studies¹⁻³ have attacked the problem in quite different ways. This month in *Paleoceanography*, Delia Oppo and Scott Lehman¹ publish the results of a monumental effort documenting the longest and most detailed record yet of surface and deep-ocean circulation changes in the subpolar North Atlantic. Earlier, also in *Paleoceanography*, M. Maslin and others² used species and isotope compositions of North Atlantic planktonic foraminifera to model changes in surface water densities and their effects on the conveyor. Three months before that, I. N. McCave and others³ had published evidence for changes in the speed of ocean circulation at intermediate and deep levels in the North Atlantic, based on grain sizes of deep-sea sediments.

All three studies concluded that sudden, recurring changes in the operation of the Atlantic Ocean's conveyor did indeed occur during the last glaciation. The work by Oppo and Lehman, in fact, comes very close to finding the long-sought link between the operation of the conveyor and the dramatic, rapid shifts of glacial climate that are documented in the Greenland ice cores.

According to Maslin and his colleagues², between the present and about 45,000 years ago there were only four times when sea surface densities were high enough to permit downward convection of surface water and formation of North Atlantic Deep Water (NADW), the deep southward-flowing limb of the conveyor. In between, they argue, were long periods when sea surface densities were so low that production of NADW was impossible, and the climate of the North Atlantic was in a polar deep-freeze. They suggest that the reduction of surface water densities was largely due to melting of icebergs released during the North Atlantic's Heinrich events, thereby blaming the frigid climates on massive surging of ice in Hudson Strait, the presumed origin of the Heinrich ice-rafting events.

They leave unexplained, though, many other prominent coolings of the North Atlantic's sea surface, such as those documented in deep-sea piston core Vema 23-81 (see figure), and correlated with the temperature records from Greenland ice cores⁴. Moreover, the record from Vema 23-81 demonstrates that even during the Heinrich events, coolings began 1,000 to 2,000 years before the arrival of the Heinrich iceberg armadas (part *a* of the figure), indicating that the massive discharges of ice from Hudson Strait only amplified a climate shift that was already under way⁵.

In sharp contrast, Oppo and Lehman¹ find a series of millennial-scale changes in production of NADW through the entire glacial cycle (part *b* of the figure), through part of marine isotope stage 5 and even into the previous glacial cycle, isotope stage 6. Their evidence comes from changes in the isotope ratio in benthic foraminifera ($\delta^{13}\text{C} = (^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}} - 1$). That measurement is thought to be a proxy for deep-water nutrient content which presumably varies with the strength of NADW production (that is, with the relative amount of downward-convecting surface water in the North Atlantic). They found in addition that the decreases in NADW production tended to coincide with coolings in the record of surface temperatures in the same core, especially after about 40,000 years ago, demonstrating for the first time that the two were closely connected through much of the glacial cycle.

Although Oppo and Lehman did not attempt to compare their results with the climate records in Greenland ice, they did compare their records with the ocean temperatures measured in Vema 23-81 (see figure), pointing indirectly to a possible link between the operation of the con-

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veyor and the jumps in air temperature above Greenland. Interestingly, they found that the shifts in NADW production are no more marked during Heinrich events than they are during other prominent coolings. They were not able to find,

veyor than that documented by Oppo and Lehman.

McCave and his colleagues³ have taken a different and ingenious approach to inferring changes in the operation of the conveyor. Arguing that nutrient proxies,

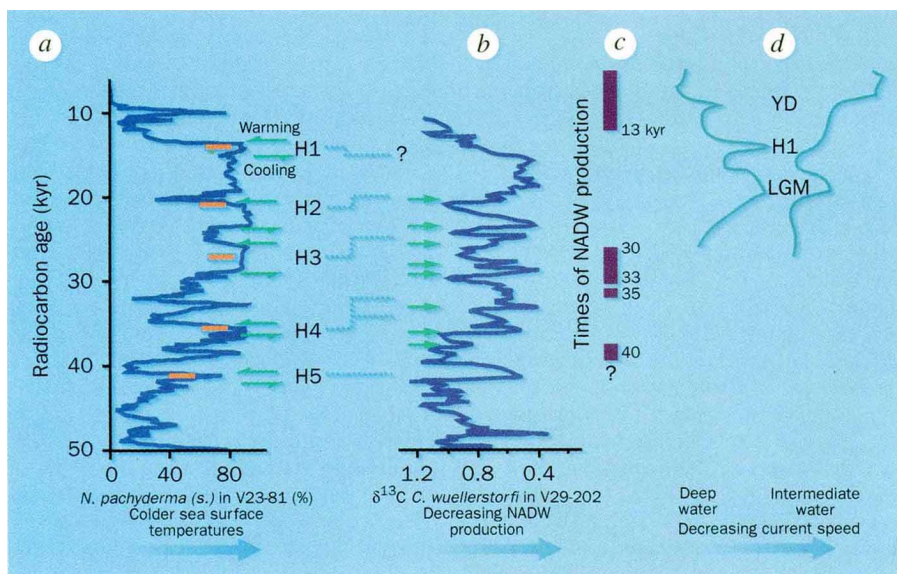
is, when NADW production is weak or switched off, deep current speeds are sluggish; when NADW production is strong, deep currents are vigorous.

Perhaps the most intriguing result of their study is a comparison of circulation speeds of deep (more than 2,000 m) and intermediate (1,000 – 2,000 m) water masses, made by measuring grain-size shifts in cores from different water depths. They found an overall inverse relation between circulation speeds of the intermediate and deep water masses (part *d* of the figure). That relation was predicted in earlier studies from both proxy measurements and modelling of ocean circulation^{7,10,11}. The implication is that the conveyor is never really switched off but only changes its mode of operation from one dominated by a vigorous production of NADW (giving a warm North Atlantic) to one dominated by production of intermediate water (cold North Atlantic).

In spite of their differences, these three studies combine to bring us closer to an understanding of the ocean's role in rapid climate change. There seems little doubt that the Atlantic's conveyor is just as unstable and prone to erratic behaviour as circulation models have suggested. Today the conveyor's mode of operation is dominated by a vigorous production of NADW which sustains a warm North Atlantic, but the clear message is that the conveyor is not likely to remain in that mode.

What is far less clear is what causes the sudden changes in the conveyor, and that uncertainty prevents us from making reliable predictions about the conveyor's operation in the near future. If a large injection of glacial meltwater is the only mechanism capable of switching the conveyor into a different mode, then the possibility of mode switches in the near future may be remote. On the other hand, if mode flips can occur independently of glacial ice, then the odds of a climate jolt in the near future might be much higher. The key to sorting this out lies in reconstructing the conveyor's behaviour during the most recent time of minimum ice volumes — the Holocene. □

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a, Abundances of *Neogloboquadrina pachyderma* (s.), a species of planktonic foraminifera, which is a proxy for polar-like water conditions measured in deep-sea core Vema 23-81 from the eastern North Atlantic. Solid bars are layers enriched in detrital carbonate, the indicator of arrival of iceberg discharges from Hudson Strait during Heinrich events. The short arrows mark rapid coolings and warmings of the ocean surface surrounding Heinrich events. The coolings consistently precede the deposition of layers containing the Heinrich iceberg debris. Data are from refs 4 and 5. *b*, Record of $\delta^{13}\text{C}$ in benthic foraminifera measured in deep-sea core Vema 29-202 from south of Iceland. Arrows mark prominent shifts in $\delta^{13}\text{C}$, which Oppo and Lehman¹ interpret as evidence of sharp reductions in the production of NADW. *c*, Estimates of times when NADW was forming, based on estimates of sea surface densities by Maslin, Shackleton and Pflaumann² in cores from the eastern and central North Atlantic. *d*, Estimates of the relative speeds of deep (>2,000 m) and intermediate (1,000 – 2,000 m) water circulation, made by McCave, Manighetti and Beveridge³ on the basis of grain sizes in silt measured in cores from the eastern and western North Atlantic. Data are from ref. 3.

though, the large reduction in NADW production that others have documented during the first Heinrich event⁶. Also, just after Heinrich event 5, NADW production was apparently quite low, while at the same time surface water underwent an abrupt, large warming, the opposite of the expected relation between surface warming and NADW production (compare parts *a* and *b* in the figure).

Why the results in refs 1 and 2 differ so markedly is unclear, but part of the answer may lie in the use by Maslin and his colleagues of summertime proxies for a process (surface water cooling and downward convection) that is more likely to be most effective during the autumn and early winter⁷. Or the differences may reflect uncertainties in calculating sea surface temperatures from faunal assemblages, which is an essential step in the procedures used by Maslin and colleagues. Whatever the reasons, the earlier study seems to give us a rather more blurred view of the operation of the con-

veyor such as those used by Oppo and Lehman, may be sending confused messages about deep ocean circulation, they attempt to identify changes in the speed of deep circulation from measurements of grain sizes in piles of sediment known as drifts. Following ideas put forward in earlier studies^{8,9}, they argue that because drifts are sites where bottom-flowing currents have a strong influence on silt-sized sediment (10 to 63 μm), changes in the grain size of that sediment, relative to that of the input sediment, should reflect relative changes in the strength of those currents.

As they anticipated, their analyses reveal prominent changes in current speeds at times of well documented climate shifts, such as the return to near-glacial conditions during the Younger Dryas, the first Heinrich event and the Last Glacial Maximum (part *d* of the figure). The changes in circulation speeds of deep water that they found are consistent with the shifts in production of NADW documented by Oppo and Lehman: that

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Painful connection for ATP

Charles Kennedy and Paul Leff

THE importance of adenosine 5'-triphosphate (ATP) in intracellular biochemistry is classical textbook stuff: it was discovered in 1929 and is the energy source for every living cell. By contrast, realization that ATP also has extracellular functions, through interaction with cell-surface receptors, has grown only recently. Papers by Chen *et al.*¹ and Lewis *et al.*² (pages 428 and 432 of this issue) add to the evidence of the extracellular actions of ATP, and point to the intriguing possibility that it is involved in the generation of pain signals.

In the past ten years it has become clear that ATP is released as a cotransmitter with noradrenaline from sympathetic nerves innervating blood vessels and visceral smooth muscle, and with acetylcholine from parasympathetic nerves controlling tissues such as the urinary bladder³. It has since also been shown to have neurotransmitter action at neuro-neuronal synapses in sympathetic ganglia⁴ and the brain⁵. When released from nerves, ATP acts at the P2X subtype of P2 purinoceptors^{6,7}, two of which — P2X₁

and P2X₂ — were cloned last year; they proved to be ligand-gated cation channels^{8,9}, and their messenger RNA is widely distributed throughout the body.

Chen *et al.* and Lewis *et al.* now describe a third P2X purinoceptor subtype, P2X₃. This subunit forms functional channels when expressed on its own, and can also form a heteromultimer with the P2X₂ (but not the P2X₁) subunit, thereby constituting a novel channel phenotype. Furthermore, P2X₃ has a highly restricted distribution, being selectively expressed at high levels in sensory C-fibre nerves running into the spinal cord, many of which are receptors for painful stimuli (nociceptors) — hence the potential connection between ATP and pain.

P2X purinoceptors are 397–492 amino acids long and have a predicted structure of two short intracellular domains, two transmembrane-spanning regions and a large extracellular domain. This is similar to the structure of epithelial sodium channels¹⁰ and putative mechanosensitive channels in *Caenorhabditis elegans*^{11,12}, although these proteins and the purinoceptors have no sequence homology. The P2X₃ subunit has 43 per cent and 47 per cent homology with P2X₁ and P2X₂ respectively; interestingly, ten cysteine residues are conserved in all three subtypes, so their tertiary structure may also be conserved.

When functionally expressed, the P2X subunits give rise to ATP-gated cation channels which have distinctive pharmacological and kinetic properties. P2X₁ and P2X₃ are activated by α,β -methylene-ATP and desensitize rapidly, whereas P2X₂ is not sensitive to α,β -methylene-ATP and when activated by ATP desensitizes slowly. However, Lewis *et al.*² noted that the currents activated by ATP in sensory neurons, the source of the P2X₃ subunit, do not fit either of these profiles — they are sensitive to α,β -methylene-ATP, but desensitize slowly. *In situ* hybridization shows the presence of all three subunits (plus a fourth subunit, the sequence of which is not yet published) in neurons, but not glia, of the nodose and

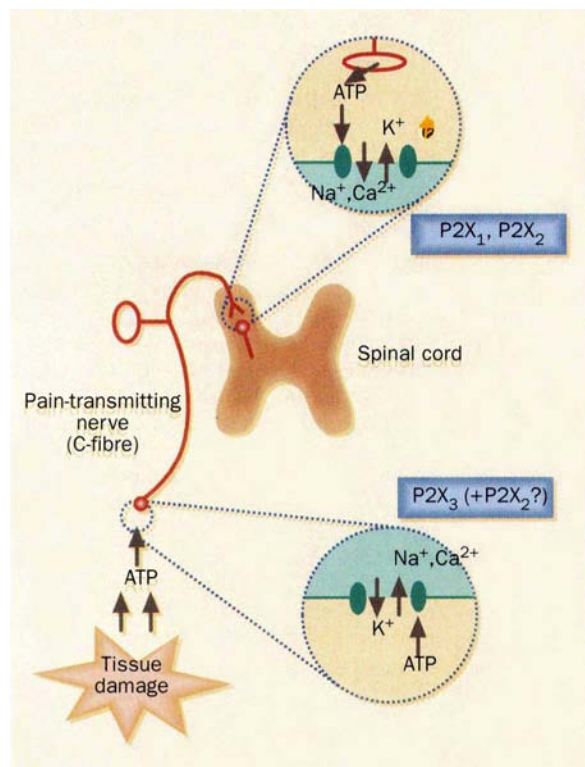
dorsal root ganglia. Therefore, they investigated whether co-expression of two different P2X subunits could lead to a novel current phenotype.

It turned out that only the P2X₂ and P2X₃ subunits seem to form a heteropolymer, with properties similar to the currents seen in sensory neurons (activation by α,β -methylene-ATP and slow desensitization). All other combinations could be accounted for by addition of the two individual sets of channels. Within the heteromultimer, P2X₂ seems to control the kinetics of the current, whereas P2X₃ determines the receptor's pharmacological properties. This is the first direct evidence that P2X purinoceptors are multimeric and, furthermore, can form heteromultimers similar to those in nicotinic and glutamate receptors and voltage-gated ion channels.

Messenger RNA for P2X₁ and P2X₂ crops up in many tissues throughout the body^{8,9}, whereas that of P2X₃ has been found only in the dorsal root and trigeminal ganglia and at very low levels in the pituitary gland. Pretreatment of dorsal root ganglia with capsaicin substantially reduced the levels of P2X₃ mRNA and, by using nociceptive neuron-associated markers, Chen *et al.* confirmed that the P2X₃ transcript is expressed selectively in small-diameter neurons corresponding to C-fibre afferents, most of which are nociceptive.

This highly selective localization of P2X₃ suggests therapeutic possibilities for the development of analgesics. Because P2X₃ (and P2X₂) subunits are present in the cell body of nociceptive neurons, they may also occur in their peripheral terminals and so be a target for extracellular ATP released from the cytoplasm of damaged cells. According to this model, ATP would depolarize the sensory nerve terminals, initiating a nociceptive signal (see figure).

ATP is also excitatory at a population of neurons in the spinal dorsal horn¹³ and may therefore act as a neurotransmitter at the central terminals of sensory neurons (see figure). The P2X₃ purinoceptor is probably not involved here, as its mRNA



Possible roles for ATP in the generation of pain signals. ATP is released from the cytoplasm of damaged cells into the extracellular space, where it may stimulate P2X₃ subunits, either alone or in combination with P2X₂, leading to depolarization of nociceptive nerve-fibre terminals and so initiation of a nociceptive signal. ATP may also be a neurotransmitter from the central terminals of these nerves. P2X₃ subunits are not present postsynaptically in the dorsal horn of the spinal cord; instead, ATP may act here at P2X₁ and P2X₂ purinoceptors (or both).

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is absent from the spinal cord, but P2X₃ located presynaptically on the central terminals of C-fibres may be. In contrast, mRNA for both P2X₁ and P2X₂ occurs in the spinal cord^{8,9}.

This model can explain the induction of pain by exogenous ATP in a human blister-base model¹⁴, and is consistent with reports that P2X antagonists are analgesic in animals^{15,16}. Clearly, if ATP, and more specifically P2X₃ purinoceptors, are involved in nociception, then the development of an antagonist selective for P2X₃ could prove useful in pain relief. Lack of P2X₃ in other tissues could confer a degree of specificity, leading to fewer side-effects.

The pharmacological detection and delineation of P2 purinoceptor subtypes has been severely hampered by the inadequacies of the available ligands, such as the susceptibility of many agonists to

metabolism by extracellular nucleotidases⁷, and the almost complete absence of selective antagonists. So their definition by recombinant biology^{1,2,8,9} constitutes something of a breakthrough. Other subtypes of P2X are also likely to be identified, as ATP activates ligand-gated cation channels in the heart, skeletal muscle and epithelial cells, yet mRNA for the three known subunits is absent from these tissues. The unequivocal structural subtyping of these receptors and their differential distribution means that the discovery of selective therapeutic agents for pain relief is a realistic goal. □

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GRAVITATIONAL DYNAMICS

Universal twists

Stacy McGaugh

THE dynamics of self-gravitating stellar systems and the large variety of complicated and beautiful manifestations thereof observed in galaxies has long fascinated and befuddled astronomers. Progress in understanding the origin and endurance of such features as spiral arms, galactic warps and related phenomena was discussed at the conference* which marked the sixtieth birthday of Professor Donald Lynden-Bell of the University of Cambridge. Although general enough, the conference title does not do justice to the breadth and depth of the topics discussed, or indeed to the contributions Lynden-Bell has made to astronomy.

A favourite subtopic of his which still exercises astronomers is the dynamics of disks and the Local Group of galaxies. Gravitational instabilities in disks could be the cause of features like spiral arms, warps and lopsidedness. These features are observed to be very common in galaxies, but as discussed by many speakers, most mechanisms for exciting them are effective only for relatively short periods. Such disturbances should quickly damp out (on orbital timescales of a few $\times 10^8$ years), so why are these features so common in galaxies $\sim 10^{10}$ years old? Some source of continuing excitation is required (S. Tremaine, Canadian Inst. Theoretical Astrophysics, Toronto), and it is not yet obvious which of the various suggested mechanisms is responsible, although some have been ruled out. Possibilities that remain viable include a Coriolis force

from a twisting halo of dark matter or the rattling induced by a halo composed of massive ($\sim 10^6$ solar mass) objects (J. Ostriker, Princeton Univ.) or tidal interactions between galaxies (A. Toomre, Massachusetts Inst. Technol.).

The disk of our own Galaxy has a quite substantial warp indicated by a flaring of gas which sets in around the edge of the stellar disk. Our own Galaxy resides in a group and is closely interacting with two much smaller satellite galaxies, the Magellanic Clouds. But it seems that this particular interaction is not strong enough to cause the observed warp. A more massive or nearer neighbour might suffice, and just last year a new dwarf galaxy, Sagittarius, was discovered very near to the far edge of the disk of the Milky Way. This led to the bold suggestion (D. N. C. Lin, Univ. California, Santa Cruz) that perhaps this recently discovered galaxy is responsible for the observed warp. Although a warp could be generated in principle by the close passage of an object on an orbit consistent with that of Sagittarius, initial reaction to this idea was sceptical. The sticking point is that to induce the observed warp, a greater mass (by at least a factor of three) is required than is obviously provided by Sagittarius. Although there are substantial uncertainties in the estimates of the mass of Sagittarius, most relevant systematic effects are likely to lead to an overestimate rather than an underestimate. Nonetheless, dwarf galaxies are generally believed to be associated with relatively more dark matter than brighter giants, and after all, something must be doing the damage.

An interesting result pertaining to the Local Group as a whole questioned the venerable timing argument. Given the mass of the main players (our own Galaxy and the Andromeda nebula), it is possible to use orbital dynamics to constrain the age of the Universe. But the usual calculation makes the assumption that this occurs in isolation. Although asymptotically true, this is not really the case from the beginning of a homogeneous universe. Accounting for this with a clever analytical approach, Lynden-Bell's student A. Whiting (Univ. Cambridge) argued that the age might be overestimated by a factor of up to $\sqrt{2\pi/3}$. Although the real effect may be smaller, this could help to reconcile current estimates of the Hubble constant, and the relatively low age of the Universe inferred therefrom, with one of the traditional arguments for a higher age.

The conference concluded with a vigorous discussion (Lynden-Bell) of the cosmological implications of Mach's principle. This essentially states that local inertia is determined by the distribution of mass-energy throughout the Universe. The apparent causality problems of this statement (so simply put) kept Einstein confused for some time. In his own words, "These were errors of thought which cost me two years of excessively hard work".

In essence, Mach's principle notes that it makes no sense to measure acceleration in some mystical absolute space, but only with respect to a remote frame of reference such as that defined by the distant stars. In this way Mach devastatingly criticized Newton's absolute space without providing a usable alternative. This has cosmological implications because it means we can never know the appropriate boundary condition to apply to Einstein's equations for the entire Universe. How can we presume space to be asymptotically flat, or to have any other arbitrary geometry in the absence of matter?

Like Einstein and Wheeler, Lynden-Bell feels that imposing the flat-space boundary condition at infinity is unsatisfactory, so that the only solution which makes sense is no boundary and so no boundary condition at all. The only symmetrical Universe for which this is the case is a closed one ultimately fated to recollapse. Lynden-Bell argues that space-time without mass-energy does not obey the no-boundary condition and therefore does not exist. In his world space-time is caused to exist by energy. Whether the Universe is bound to follow this powerful theoretical argument is another matter, but Donald has been right more than once and we would do well to heed him now. □

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The sound of silence

Thomas Perlmann and Björn Vennström

THE nuclear receptors for steroids, thyroid hormone and retinoic acids are ligand-dependent transcription factors that activate transcription through specific DNA-binding sites in their target genes. But what are these receptors doing in the absence of their ligands? This is the question addressed by papers by Hörlein *et al.*¹, Kurokawa *et al.*², and Chen and Evans³ (pages 397, 451 and 454), which deal with a less recognized feature of such receptors — their ability to silence basal transcription.

Factors referred to as co-activators are thought to serve as bridging molecules, or adaptors, between the receptors and the basal transcriptional machinery, thus mediating activation of transcription. The three papers^{1–3} describe two newly discovered factors that interact with receptors, but rather than activate transcription these factors repress it. So we can add a new category of molecules — co-repressors — to the growing list of receptor-modulating proteins.

The ability to repress basal transcription was first observed in the thyroid hormone receptor (TR) and in its viral oncogenic counterpart v-ErbA, but later also in receptors activated by retinoic acid (RARs)^{4–6}. Although repressing activity has at times seemed elusive, data hinting at the existence of silencing factors provided the incentive for continued research^{7,8}. Now, with the characterization of two distinct silencing factors, the phenomenon of trans-repression appears to rest on solid ground.

Both factors were identified by two-hybrid screening in yeast and are shown to interact with both TR and RAR. Hörlein *et al.*¹ and Kurokawa *et al.*² refer to their factor as N-CoR (nuclear receptor co-repressor), and Chen and Evans³ use the name SMRT (silencing mediator for retinoid and thyroid hormone receptors). A partial sequence of N-CoR was previously published as a receptor-interacting factor with unknown function⁹. N-CoR and SMRT show some homology in a region that includes their receptor-interacting domains, implying the existence of a family of related co-repressor molecules.

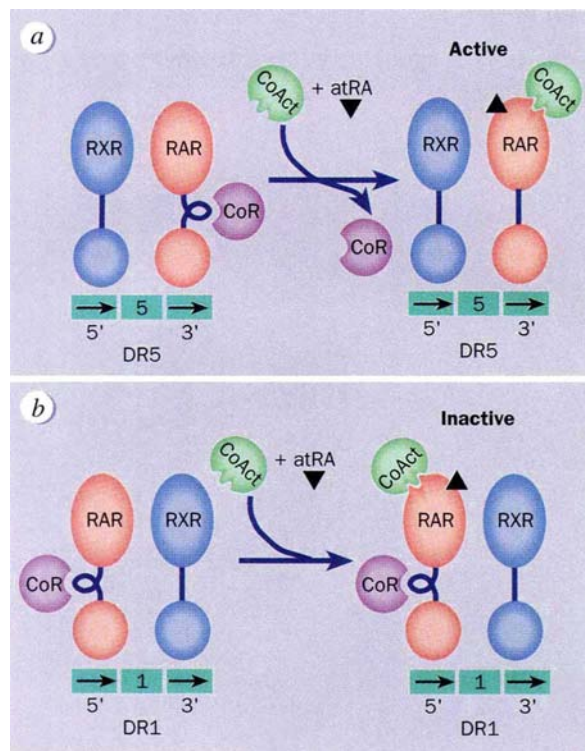
N-CoR and SMRT are reported to mediate repression, but is there strong evidence linking these factors to the silencing effects? The answer is yes. First, N-CoR and SMRT interact with unliganded receptors and are released upon ligand binding^{1,3}. This is exactly what would be expected: TR and RAR repress transcription in the absence of ligands but activate it very efficiently in their presence.

Second, there is previous evidence that the flexible hinge region which connects the DNA-binding and ligand-binding domains of receptors is important for silencing. All three papers show that mutations introduced into this region of TR or RAR abolish transcriptional silencing as well as interaction with the co-repressors.

Third, the biological significance of this interaction is elegantly demonstrated by Chen and Evans³. A mutation originally found in the hinge region of a defective version of v-ErbA, the oncogenic counterpart of TR, inactivates the oncoprotein and abolishes its transcriptional repressing activity^{10,11}. Interestingly, TRs containing this mutation are defective in repression and are unable to bind SMRT, thus providing a link between silencing and oncogenic transformation. Finally, being mediators of repression, co-repressors would be expected to contain an intrinsic silencing activity. When either N-CoR or SMRT is recruited to promoters by fusion to a yeast GAL4 DNA-binding domain, efficient repression is indeed observed^{1,3}.

The story could have ended here, were it not for another puzzle that is clarified by Kurokawa *et al.*². Many nuclear receptors, including RAR, bind as heterodimers with the receptor for 9-*cis* retinoic acid, RXR, to specific DNA sequences organized as tandem repeats. Accordingly, the RXR-RAR heterodimer activates transcription very efficiently from direct repeats spaced by five nucleotides (DR5; see figure). RXR bind 5' and RAR 3' on such elements. In contrast, the polarity of the complex is reversed on direct repeats spaced by one nucleotide (DR1); RAR is unable to activate on such elements although it can bind ligand, and as a consequence the heterodimer acts as a potent repressor when the polarity of the receptors is reversed¹².

In this same paper, Kurokawa *et al.*¹² suggested that binding in the 5' position imposed a non-permissive conformation on RAR, thus blocking its activation. Kurokawa *et al.*² now show that co-activators are recruited equally well on both DR5 and DR1 elements in the presence of ligand. N-CoR, however, turns out to be the key explaining the differences in activation — whereas N-CoR is released from the DR5 element in the presence of ligand, it remains tethered to the complex on DR1, making it unable to activate on these elements (see figure). In support of this view, the authors also report that mutated RARs, unable to interact with N-CoR, can activate transcription from DR1 elements (see figure). From these data it



a, Heterodimers between the retinoic acid receptor (RAR) and retinoid-X receptor (RXR) bind to direct repeats spaced by five nucleotides (DR5), with RXR (blue) bound in the 5' and RAR (red) in the 3' position. In the absence of all-*trans* retinoic acid (atRA, black triangle), co-repressor (CoR, purple) is associated with the complex thus mediating repression. Ligand will induce both the dissociation of co-repressor as well as the recruitment of co-activator (CoAct, green), and the complex will be active. b, On DR1 elements, by contrast, the orientation is reversed, so that RAR is bound in the 5' site. In this configuration the co-repressor is unable to dissociate from the complex thereby preventing transcriptional activation. The model implies that co-repressors are dominant over co-activators.

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would seem that a receptor such as RXR, which does not interact with N-CoR¹, would also activate in the 'non-permissive' 5' position of a direct repeat. Indeed, although binding 5' in a heterodimer with the orphan receptor NGFI-B on a DR5 element, RXR efficiently activates in response to its ligand, 9-*cis* retinoic acid¹³.

What are the functions of silencing? Kurokawa *et al.*² suggest that one of them may be to increase target-gene specificity to certain elements (such as DR5 in the case of RAR). It will also be interesting to learn whether N-CoR, SMRT or other co-repressors interact with receptors such as the orphan COUP-TF, known to be a very

potent repressor¹⁴; and whether N-CoR and SMRT are involved in some receptors' inhibition of transcription in the presence rather than absence of ligand.

Clearly, much remains to be done. But with the identification of the co-repressors, and the characterization of their activities, we have the essential tools to help understand the physiological role of trans-repression. □

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SEXUAL SELECTION

Déjà vu all over again

Mark Kirkpatrick and Nick Barton

SUCH philosophical luminaries as Heraclitus and Jerry Garcia have viewed our universe as a cascade of never-ending cycles. A similar perspective emerges from a mathematical model by Iwasa and Pomiankowski, described on page 420 of this issue¹, which suggests that sexual displays such as the peacock's tail can evolve "by a commodius vicus of recirculation"². The model offers a hypothesis for one of sexual selection's great mysteries: the rapid evolutionary divergence of these displays between closely related species (for example between peacocks and their pheasant relatives).

In Iwasa and Pomiankowski's model, cycles are driven by a delicate balance between two of the evolutionary forces that act on mating preferences: direct selection and indirect selection. Direct selection occurs whenever preference genes affect survival or reproduction directly, which they may do in varied and subtle ways³⁻⁵. The new model assumes that females with stronger preferences suffer greater costs (from predation, for example) while searching for males. Indirect selection on preferences occurs when preference genes become associated with other genes that are themselves directly selected. Such a genetic correlation between preferences and traits is a natural consequence of mating, because genes that cause females to prefer males carrying particular genes at other loci will tend to find themselves with those genes in the offspring.

In a verbal argument as famous for its obscurity as its prophesy, R. A. Fisher⁶ pointed out in 1930 that the male trait itself could drive indirect selection of preferences. Stronger preferences select directly for more exaggerated male traits, which in turn select indirectly for yet stronger preferences via the correlation. The outcome can be a 'runaway process',

an orgy of self-reinforcing exaggeration of both the preference and trait. Fisher's arguments were finally confirmed in most respects by Lande⁷ in 1981, from whose analysis it emerged that the genetic correlation between preference and trait must be larger than a critical threshold for a runaway to be triggered. It is a tacit assumption of Iwasa and Pomiankowski's model that the correlation is larger than this threshold. A final ingredient of their model follows another of Fisher's suggestions: the intensity of natural selection opposing further exaggeration of the male trait increases very rapidly when it is far from the point that maximizes survival.

What then happens? Evolution of the trait and preference proceeds in two phases (see Fig. 1 on page 421). First a runaway is initiated, causing rapid exaggeration of the trait and preference. This phase is brought to a close when the trait becomes so extreme that the accelerating intensity of natural selection finally inhibits further excesses. At this point, direct selection on the preference becomes stronger than indirect selection. The preference is slowly dragged back towards the value that maximizes female survival, taking the trait with it. But just as this point is reached, a runaway is triggered again. And off the trait and preference go, like Sisyphus chasing his rock. Isolated populations experiencing these dynamics will soon fall out of step with one another, the authors point out, perhaps explaining the

rapid divergence of closely related species that has often been noted in sexually selected taxa.

The model illustrates features of dynamical systems that are unfamiliar in evolutionary biology, but which are well understood in other fields. The cycles involve a fast 'runaway', and a slow return towards the preference favoured by natural selection. This coupling between fast and slow processes is typical of (for example) the conduction of nerve impulses⁸ and the periodic outbreaks of insect pests⁹. It is an open question whether populations diverge, eventually to form new species, as a result of such spontaneous cycles, or whether they do so because of random genetic drift, random accumulation of mutations or in response to changing environments.

Iwasa and Pomiankowski's model demonstrates that rich dynamics can emerge in models of the interplay of natural and sexual selection. Whether cycles of sexual selection can occur in nature, however, will only be resolved after more work. The model makes the implicit assumption that the male trait experiences no natural selection when it is near the value that maximizes survival. This theo-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASON
REASONS

Tell tail — a Bulwer's wattled pheasant shows off.

retically convenient but biologically unconvincing assumption renders the trait and preference highly unstable: the threshold value of the genetic correlation that ensures a runaway is only 0.005 for the example shown the authors' Fig. 1.

With natural selection of the sort seen in nature, however, the threshold can be orders of magnitude larger. Data on natural selection in the wild^{10,11} suggest that a term like $\exp(-t^2/40G_t)$ should be included in their equation for male fitness (where t

is a male's trait value and G_t is the trait's genetic variance): the correlation threshold then rises to about 0.25, and it rises still further, to about 0.5, if the preference experiences a similar intensity of stabilizing selection as well. No cycling occurs if the correlation is below the threshold value. Instead the population settles into an equilibrium that may or may not feature extreme male displays, depending in part on whether or not exaggerated preferences are produced as a side-effect of natural selection on female behaviour. This is an important but unanswered empirical issue.

So the possibility of evolutionary cycles hinges on the size of this genetic correlation. How large do we expect it to be in nature? So far, theorists have been able to calculate the correlation only under assumptions that are simplified caricatures of the genetics and behaviours that underlie real traits and preferences. Predictions for the correlation that are robust to these poorly known details and based on measurable quantities would be useful.

A second wish is for a theory of how the genetic variation in the preference and trait changes through time — which of course requires that we understand the causes of quantitative genetic variation. Iwasa and Pomiankowski take these variances to be constants, which is reasonable given that the means of the trait and preference make excursions of only about one phenotypic standard deviation in their examples. But that amount of change is trivial compared to the differences we see between, say, the male plumages of different pheasant species. If cycles are involved in generating such diversity, we will ultimately need a theory for the dynamics of the variances when means evolve over many phenotypic standard deviations. Whether changing variances will inhibit cycles or amplify them, or perhaps throw evolution into chaos, is anyone's guess. □

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Towards an engineering era?

Charles H. Bennett and David P. DiVincenzo

ONE of the paradoxes of quantum theory is how little its strange foundations obtrude into everyday life, even while supporting an edifice of chemistry and physics that explains the existence and properties of ordinary matter in exquisite detail. To marvel at the foundations themselves — in the form, say, of the two-slit experiment or the Einstein–Podolsky–Rosen effect — one must generally visit a textbook or a laboratory. But it is beginning to appear that the foundations of quantum mechanics will find a fairly direct and visible application in the new science of information processing.

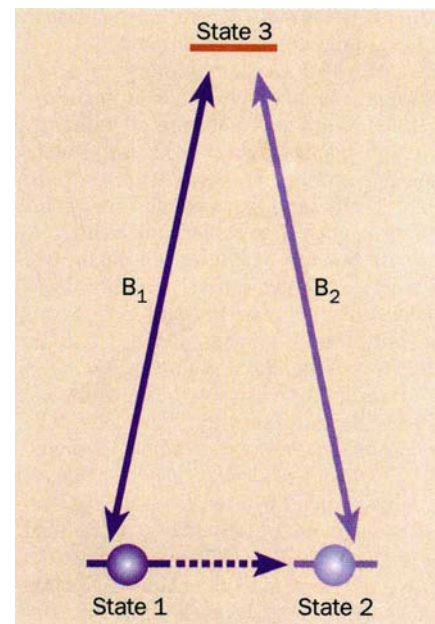
For about ten years, there have been speculations about the vast and distinctive powers a quantum computer would have if one could ever be built. Now it appears that the subject of quantum computing is emerging from this visionary prehistory into a distinct 'experimental' era. Powerful algorithms exploiting the unique capabilities of a quantum computer are now widely understood, and systematic searches for new algorithms are under way. On the hardware side, the implementation of the logic gates of a quantum computer is now, at some rudimentary level, within the purview of well established experimental physics. It is still too early to tell when or whether quantum computing will advance to a third, 'engineering' era of routine practical application.

At a summer workshop of quantum computation*, the talks, while still including 'visionary era' discussions of Bell's inequalities, quantum cellular automata and the 'many-worlds' interpretation, had moved on to include concrete discussions of experimental realizations of quantum logic gates — to the extent that the University of Innsbruck group unabashedly presented a "realistic model of a quantum computer" (T. Pellizzari; ref. 1).

Their scheme for implementing quantum logic builds on the details of some of the hottest trends in atomic spectroscopy, in particular a technique called 'dark-state transfer of Zeeman coherence'. A Zeeman-coherent atom is one in which the wavefunction is only non-zero for one of the members of the ground-state multiplet (containing Zeeman fine structure). This wavefunction can be moved to the other member of the multiplet if the atom is exposed to two optical beams which couple these two ground-state levels to an excited state (see figure). The rule is first to turn beam B_2 on, which leaves the system in state 1, but strongly couples states 2 and 3. When B_1 is turned on next, the

system is transferred from 1 to the 2–3 complex, but, remarkably, because of quantum interference the amplitude of the wavefunction in the excited state 3 always remains very small. Then if B_2 is turned off, the system wavefunction is entirely in state 2; turning off B_1 completes the process.

This kind of spectroscopy has many advantages as a basic step in quantum



In dark-state spectroscopy, a quantum system passes from state 1 to state 2 by mixing with an excited state 3 using coherent radiation (modes B_1 and B_2).

computation. The instantaneous state is always 'dark': that is, it cannot suffer spontaneous emission from the excited state, as its probability density for being in this state is vanishingly small (if the beams are turned on and off slowly enough). Thus the system always remains in a pure quantum state. If one of the beams B_2 is a localized cavity mode in a superposition of zero- and one-photon states, then the spectroscopy serves to map that superposition into the same superposition of the two Zeeman sublevels. It is this transfer capability which the Innsbruck workers have extended to solve the hardest problem of physical quantum logic gate design: moving a quantum state undisturbed from one subsystem (atom) to another.

Several experiments have proved that different quantum bits (or 'qubits') can be made to interact controllably, in such a way that a rudimentary quantum logic gate is formed. In the work of C. Monroe and collaborators², it has been successfully shown that the low-lying states of beryl-

* *Quantum Computation*, Institute for Scientific Interchange, Turin, Italy, 25 June–7 July 1995.

lithium ions in a Paul trap can be coupled spectroscopically to phonons (not photons) — vibrational quanta of the trapped ions. Their work, inspired by a theoretical proposal by Cirac and Zoller³, demonstrated the successful operation of a quantum exclusive-or or controlled-NOT gate, which flips one qubit conditionally on the state of another qubit, with an overall reliability in the initial experiments of about 80 per cent. In another experiment reported at the Turin meeting (Q. A. Turchette, California Institute of Technology), the roles of the quantum information carriers are reversed: cavity quantum electrodynamic systems are studied in which the interaction between different photon states is mediated by an atomic state (in this case, of a caesium atom in a beam passing through a high-finesse optical cavity). The group have determined indirectly that this set-up would provide an atomic-state dependent phase-shift (up to 16° in one experiment), from which logic gates like the exclusive-or could be built up.

These last few examples are illustrative of a general theme that pervaded both theoretical and experimental discussions at Turin (and elsewhere): a lot can be done in systems where quantum states can be controllably interconverted from one physical form to another. Fundamentally new approaches to experiments involving the Einstein-Podolsky-Rosen (EPR) paradox, which have implications for real quantum cryptography, may come out of these discussions. An EPR pair (made out of two photons, say) is usually a fleeting thing, lasting as long as it takes the constituents to traverse an experimental apparatus to a detector. Suppose, instead, that the photon quantum state could be 'swapped' using dark-state spectroscopy into a long-lived quantum state like the Zeeman sublevels of an atomic ground state; such a state is potentially some six orders of magnitude longer-lived than the original photon state. This capability of storing two halves of an EPR pair for a long time is a prerequisite for many novel forms of quantum information transmission.

One interesting protocol introduced at Turin is a 'purification' process. Suppose a collection of particles, each of which is half of an EPR pair, is received and stored, but that some corruption of the quantum state (losses, say, or decoherence) occurs during transmission or storage. In purification (outlined below), this collection of particles is processed so as to leave a smaller number of particles, each of which is a member of a now nearly perfect EPR pair. Such a technology would open up a variety of possibilities. It would, for instance, make possible the separation of EPR pairs by much greater distances, permitting quantum cryptography and teleportation⁴ to be practised over distances much farther than a typical photon

can travel through an optical fibre without loss.

The actual processing involved in purification is a sequence of quantum-coherent exclusive-or gates supplemented by classical messages between the two parties; thus, purification is itself a kind of quantum computation. Workers at Turin were encouraged that other quantum computations of fundamental interest exist besides the famous algorithm suggested by Peter Shor for factorization of integers. It is also heartening that purification and other error-reduction schemes, in particular a very recent technique described by Shor⁵, as well as schemes for quantum data compression, become useful at scales far below quantum factorization: Shor's algorithm surpasses classical factorization methods only for quantum computations involving perhaps millions of quantum gate operations, whereas for purification the number of gates may only be in the tens. Workers in the field see even tens of gates as a monumentally difficult job experimentally, and no one has any idea when the 'engineering' era will be entered even for so simple a computation. Needless to say, Shor factoring is much, much more remote. Still, when Shor offered a wager during his seminar at Turin that the first factoring of a 500-digit number would be accomplished on a quantum computer, there were no takers on the other side.

Although Rolf Landauer was not in attendance at Turin, his work⁶ throughout the 'visionary era' of quantum computing exercised an influence over much of the present work. He has constantly challenged the 'visionaries' to show a workable path to the future, sceptically pointing out that abstract Turing-machine Hamiltonians do not make an apparatus, that dissipation and disorder are inherent in physical systems and cannot be ignored, and that computers would not be what they are without error correction. As a result, Landauer's points now serve as accepted terms of admission to the 'experimental era'. The serious workers in the field may not yet be conclusively demonstrating success, but they are proposing real apparatus, not abstraction; they are worrying about and calculating the effects of dissipation and disorder; and they are struggling with the meaning of error correction in a quantum-coherent system. □

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Air craft

SOME migrating birds fly thousands of kilometres, often over water without insects to feed them or atmospheric thermals to lift them. Where do they get the energy? Daedalus reckons that they extract it from the air.

The wind, he points out, blows in turbulent flow; it is full of eddies. A clever enough bird could pick a path through just those regions where the air happened to be rising, and moving in the direction it wanted to go. It could even exploit eddies smaller than itself. A bird's feathers are all coupled fairly independently to its frame. A feather meeting a local updraught may curve to trap it, while one experiencing a downdraught bends out of its way. Indeed, says Daedalus, that's why birds have feathers. They form an 'active wing' far more efficient than any static aerofoil. Birds migrate at fairly low altitudes to exploit the greater turbulence there.

So Daedalus is designing an eddy-powered flying-machine. The hang-glider principle seems appealing, but Daedalus needs something flatter and bigger, with an area large enough to capture the dilute energy of the eddies all around it. His new 'Aerosail' will spread a wide aerofoil out on bifurcating ribs from a central spine, like a leaf. Cunningly, it is a thin film of a piezoelectric polymer, such as PVDF. A dense pattern of flaps and holes all over the surface is connected by conducting paths to a central computer.

Each flap senses the local air flow, sends a signal to the computer, and receives back a voltage that bends it into or out of the slipstream. Each leading-edge element will sample its bit of atmosphere, and set up the regions behind to exploit it. Over each patch of wing, an updraught will be captured, holes will open to let out a downdraught, elements of tailwind will be caught and headwind dodged. Surplus energy captured by the piezoelectric elements will 'flap' the wing ripple-fashion, like a flatfish, and angle it to steer the craft.

The software for all this will be daunting. The Aerosail may even have to learn to fly by neural-net optimization, as a bird does. But then a wonderful new flying-machine will take the air. Silent, simple and elegant, the Aerosail will swoop and soar endlessly, its hundreds of square metres of film glittering in the sunlight. It will live in the air as a fish lives in the sea. Remote-controlled Aerosails could carry airmail or light freight; the largest feasible sizes could just lift a human pilot. A new sport will be born — or maybe not so new. Daedalus may have stumbled on the secret of the magic carpet. David Jones

Small genomes for better flyers

SIR — Genome size (nuclear DNA content) is both lower and more uniform in birds than in other tetrapods. The mean DNA content for 165 avian species was reported to be 2.82 ± 0.33 pg per cell¹, as opposed to a mean of about 8 pg per cell in mammals^{2,3}. Because avian genomes are smaller on average than those of their closest relatives the reptiles, as well as those of amphibians and mammals, it seems likely that this is a derived character rather than a primitive one¹. One way in which a reduction of genome size has been achieved is by the loss in birds of the pattern of short period sequence interspersions, which is known to be correlated with reduced genome size⁴. To test whether genome size reduction in birds has extended to protein-coding genes themselves, we compared 111 introns and 141 exons, homologous between humans and chickens, for 31 genes whose sequences were available in the database.

Human introns were significantly longer than their chicken homologues (244.9 ± 82.5 base pairs; *a* in the figure). By contrast, although exons averaged slightly larger in human than in chicken (2.3 ± 1.3 base pairs), the difference was not statistically significant. The slope of the plot of logarithms of corresponding intron lengths in chicken against human (0.37) was significantly less than 1.0, indicating a negative allometric relationship (*b* in the figure). The equivalent plot of exon lengths also showed significant negative allometry, but

the slope (0.98) for exons was significantly greater than that for introns (*c* in the figure). The strong negative allometry in the case of introns indicates that DNA loss from chicken genes has occurred disproportionately in long introns.

Alignment of human and chicken introns suggested that DNA loss has occurred by multiple separate deletion events scattered throughout the intron, rather than by deletion of a single large stretch of DNA (data not shown). DNA loss in avian introns was not correlated with a significant change in nucleotide content. The mean per cent G+C in human introns was 56.5 ± 1.2 , whereas that in chicken was 54.6 ± 1.3 . On the other hand, at third-codon positions in exons, human genes were significantly more G+C-rich ($G+C = 70.4 \pm 1.3\%$) than their chicken homologues ($G+C = 67.5 \pm 1.5\%$; $P < 0.05$; paired-sample *t*-test).

It has been proposed that modern birds are all descended from one lineage that survived a catastrophic event 65 million years ago⁵. A mutation occurring during such a severe bottleneck, even a disadvantageous one, could become fixed by genetic drift⁶, but the present results suggest that genome compaction in birds did not occur through such a mutational event. The fact that numerous independent deletions in large introns have contributed to genome compaction suggests that reduction of avian genome size occurred gradually over a long period. A

pattern of persistent reduction of genome size is consistent with long-term directional selection favouring small genome size in birds.

The metabolic demands of flight may place a constraint on genome size^{7,8}. Genome and cell size are correlated in vertebrates, and avian cells are generally smaller than those of mammals⁸. As the surface-to-volume ratio of a cell is negatively related to cell volume, small cell volume permits a greater rate of gas exchange per unit volume. Further, bats (order Chiroptera) have much smaller genomes than other mammals⁹, whereas among avian families genome sizes tend to be larger in flightless birds and weak flyers than in strong flyers (data not shown). Thus, it would seem that reduced genome size is an adaptation for flight in vertebrates.

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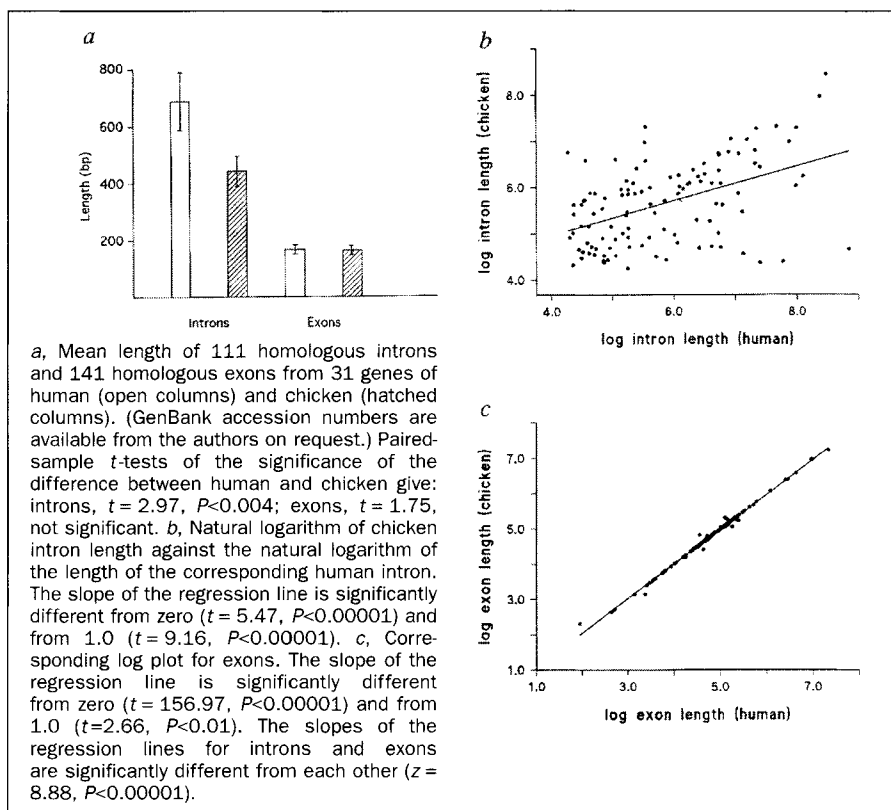
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Making a better superconductor

SIR — In a recent News and Views¹, Grant eloquently captured the excitement surrounding the recent progress at Los Alamos on ion-beam-assisted deposition (IBAD) technology. IBAD offers a fresh path for making long-length, flexible, high-temperature superconducting (HTS) wires. Here I give an industrial perspective on this development from a manufacturer of HTS wires and wire products.

At American Superconductor (ASC) we consider IBAD to be a very positive development and share Grant's enthusiasm. If manufacturing issues can be resolved, this technology using the $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ (YBCO) superconductor promises to extend the range of application of HTS wires, particularly at high fields and temperatures, beyond that expected from the deformation-processed wires based on $\text{Bi}_2\text{Sr}_2\text{Ca}_2\text{Cu}_3\text{O}_{10}$ (BSCCO).

For commercial applications, it is important to consider the engineering current density J_e , which divides the current by the full cross-section of the wire, rather than the HTS current density J_c , which divides the current only by the cross-section of the



superconducting material. Very high values of J_c have been reported by Los Alamos in the IBAD YBCO film, reaching 1.3 MA cm⁻² at 77 K and zero field over a thickness of 1.2 µm. This film was deposited on a 125-µm substrate, so that J_c is much smaller but still significant at 12,500 A cm⁻². For comparison, ASC, Sumitomo Electric and others have reported multifilamentary deformation-processed BSCCO wires reaching comparable levels² (J_c =9,000 A cm⁻²). Thus, comparisons are now much closer than press reports might suggest.

Both technologies have prospects of improving significantly, with the J_c potential of IBAD significantly higher if the substrate thickness can be reduced and film thickness increased. If the Los Alamos result were obtained on a 1-mm substrate (which is just practical for a long-length tape), J_c would exceed 60,000 A cm⁻². By comparison, if record short-length BSCCO results on peeled layers³ can be translated into multifilamentary wires with a 33% fill factor, one could expect 37,000 A cm⁻².

Another issue involves a.c. coil applications; a.c. coil losses will be high in the IBAD flat-tape configuration, while there is more flexibility to cut down these losses in a multifilamentary, twisted composite BSCCO technology. Where IBAD YBCO could really shine is in d.c. applications at higher fields and temperatures.

The real question for the future concerns ease of manufacture. Deformation-processed wires are at present being pilot-manufactured in sufficient quantity

to allow development of prototypes worldwide in such applications as motors, transmission cables and transformers. An HTS research magnet from Sumitomo Electric has recently produced a record 4-T field at 4.2 K, and an ASC HTS magnet reached more than 0.6 T at 77 K. The 77 K performance of the BSCCO wires is thus sufficient for power-transmission cables and iron-core magnets such as those in transformers. Applications at higher fields will require lower temperatures. This technology is rapidly moving towards a first generation of commercial products.

The exciting new IBAD results are on short-length samples, while early efforts at Fujikura and Sumitomo Electric to scale up the process with a reel-to-reel system are yielding metre-long tapes. There is a long way to go in scaling up manufacture, and unless deposition rates can be improved by one or two orders of magnitude, the process will be too costly for bulk application. Such progress is conceivable, though not certain, and will require research.

IBAD is an important new option for future HTS bulk wire technology, but one could expect it to be involved in a second generation of commercial products. Research investigating the manufacturing-related issues should proceed apace.

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Controversial cheetahs?

SIR — May¹ has discussed some^{2,3} of the recent reappraisals (for example, refs 2–5) of O'Brien and colleagues' evidence that the cheetah's genetic impoverishment is threatening the species' persistence^{6–8}. Some comments, however, particularly those of O'Brien⁸, may be misleading.

First, we can find little indication that the scale or causes of cheetah cub mortality in the lair (72% ($n=125$) of cubs died by emergence, approximately 73% of cub deaths due to predation⁹) were inflated by researchers in the Serengeti, as O'Brien suggested⁸. Extensive precautions were taken, minimizing vehicle tracks and the time spent watching cubs, avoiding entering lairs when a predator or cheetah mother was in sight, and moving away at night¹⁰. Statistical comparisons of litters that survived or died from different causes could not find an effect of the observer on the scale or causes of cub deaths¹⁰. Lions and spotted hyaenas in the Serengeti study site appear habituated to vehicles and generally ignore them whereas observations of predation events suggested that the cheetah mother herself gave away the position of the lair by sitting

up, entering or leaving the lair^{5,10}.

Second, although arguably no area in Africa is now 'pristine', the Serengeti National Park, with some human disturbance around its fringes, is probably no more disturbed than others. The Serengeti ecosystem has the lowest (0.004) predator/prey ratio of nine protected areas across Africa (mean 0.013, range 0.004–0.027)¹¹, and so this cannot account for high cheetah cub mortality¹. Cheetahs are not becoming crowded in protected areas³. Accurate estimates of carnivores are scant, but cheetah densities have been stable over the past 25 years in the two reserves in East Africa with appropriate data (1 per 9 km² in Nairobi National Park¹²; 1 per 42 km² Masai Mara Game Reserve; unpublished data).

Although the evidence for lack of variation at the major histocompatibility complex is suspect, we also acknowledged⁴ that the cheetah remains potentially vulnerable to pathogens. Nonetheless, few data support this thesis. The feline infectious peritonitis outbreak is poor evidence, because the response of infected captive cheetahs was not homogeneous,

with approximately half the animals surviving infection. Also, serology and vaccine trials have shown that cheetahs can recognize and respond to a range of pathogens⁴.

Finally, we would like to highlight the fact that the finding by O'Brien *et al.* of higher juvenile mortality rates in captive cheetahs than captive rodents or ungulates⁷, used as a third piece of evidence that cheetahs were suffering from homozygosity, is severely flawed^{2–5}. Analyses of mortality rates across orders, zoos and time, and without reference to causes are inappropriate. Intrinsic sources of mortality are insignificant compared with extrinsic sources, both in the wild and in captivity⁵. Moreover, deleterious recessive alleles can become purged during inbreeding¹⁴.

The history of the cheetah controversy is instructive for the way science develops. Originally, an empirical finding of homozygosity in cheetahs⁶ was used to predict consequences of inbreeding for the species. Strands of evidence gleaned from captivity and the wild appeared to support these predictions⁷. However, better analyses and empirical data fail to corroborate these predictions^{2–5}. Thus, within a 12-year period we have come full circle. Ecology can be as important as genetics, and interdisciplinary cooperation in conservation problems is essential.

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Picture credit

In "The planktonic life of octopuses" by R. Villanueva, C. Nozais and S. v. Boletzky (Vol. 377, page 107), published in the 14 September issue of *Nature*, the photograph in Fig. 1 should have been credited to Jean Lecomte, CNRS, Observatoire Océanologique de Banyuls, France. The cover picture for that issue should also have been credited to Jean Lecomte.

Suicides without sadness

Peter Tallack

Death by Design: Where Parallel Worlds Meet. A documentary film by Peter Friedman and Jean-François Brunet. *Strange Attractions/Les Films du Bouc*. 75 mins. To be broadcast in France and Germany on ARTE on 7 October, and later on public television in the United States and on RTBS in Belgium.

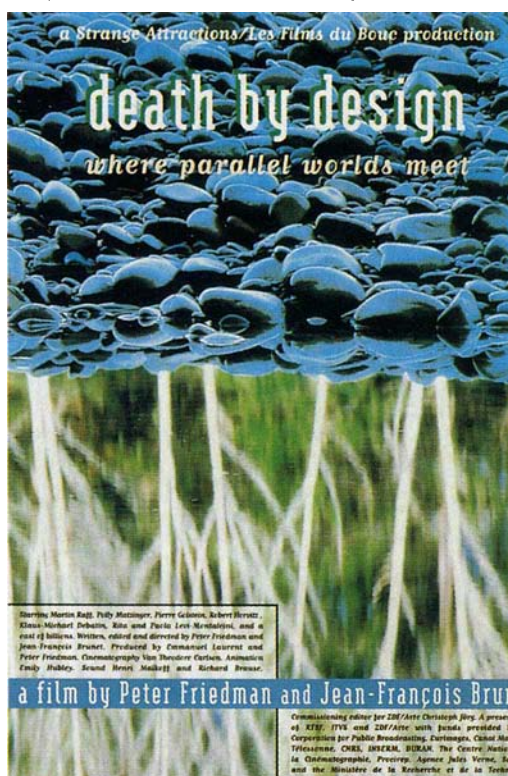
CELLS have finally made it onto celluloid. *Death by Design* is a highly original documentary about one particular aspect of their lives — their death. Made by Peter Friedman, an independent film maker, and Jean-François Brunet, a molecular neurobiologist, and sponsored by the CNRS, INSERM and the French Ministry of Research, the film truly lives up to its promise of making the screen “real and alive”. Its “cast of billions” includes a vast array of unicellular organisms as well as slime moulds, nematode worms and other such multicellular notables as Polly Matzinger, Robert Horvitz, Rita Levi-Montalcini and Martin Raff. The combination of stunning images and informed commentary provides a perfect introduction to cell biology.

All the familiar topics are here, from the Dutch merchant and self-taught scientist Anton van Leeuwenhoek, one of the first people to see bacteria and protozoa, through to the spectacular diversity of cells, and on to the origin of multicellular organisms. (Incidentally, Van Leeuwenhoek, who nearly blinded himself by looking at gunpowder exploding under a microscope, was in fact born in 1632, a century later than Matzinger claims.)

But the film's main purpose is to expose one of the more elusive qualities of cells: programmed cell death, or ‘apoptosis’. The process may be as important as cell division in generating an individual with the right number of different cell types in the right places. And because it is quick and covers its tracks, the deaths easily go unnoticed — as indeed they did during the decades when research on cell division flourished. It can regulate neuronal connections, dispose of cells that have finished their job and help to sculpt the shapes of developing organs. “Over and over again”, says Raff, “biologists have learned that this is how biology works” — it produces thousands of variants and picks the few that succeed. To illustrate the point, the film cleverly switches to a shot of the cutting room, where the producer is seen editing the sequence just shown.

Microcinematography of cells is ingeniously meshed throughout the film with parallel images of life at the human scale. A screen seething with cells blends into scenes of the New York stock exchange; and images of dying cells collapsing in on themselves cut to imploding skyscrapers.

Rarely does the film fail to deliver something memorable, whether a startling fact (up to 90 per cent of neurons commit suicide in some regions of the developing human brain), or a neat turn of phrase (“the cells are not killed — they are told to



die”, says Matzinger), or a piece of trivia (Horvitz, standing in his laboratory, estimates that he has between a billion and a trillion nematodes in the room with him), or a slapstick sequence (Harold Lloyd, the American daredevil comedian, is seen vainly trying to kill himself), or an unfamiliar image (a hundred lighted violins).

The biological dances of the living world are here portrayed as art: Sibelius accompanies a wonderful sequence of nematodes going about their business; bacteria mate to the rhythms of Bartok; and tadpoles metamorphose to the strains of a sitar raga. Great care has been lavished on presentation: some items are only a few seconds long, but are spliced with others to form a whole that, like an organism itself, transcends the sum of its parts.

There are moments of anthropomorphic excess, particularly the animated cartoon about the elimination of undesirable lymphocytes. But the philosophical limitation

of seeing cells as people is unlikely to concern the general viewer, especially when it adds humour and charm to a tricky topic. One recurring analogy to which the film perhaps gives too much credence, however, is the idea that human society is a super-organism. To be sure, cells in an organism are clones of one another, and so may commit suicide in the genetic interests of the group as a whole. But it is doubtful whether people in a city kill themselves to promote the interests of a societal super-organism. And, as is pointed out, a cell can really be said to commit suicide only in the sense that it participates in its own death; it doesn't actually decide to die.

The film is by no means all about cells.

There is also some delightful commentary on the nature of science. Why, for example, has something so fundamental as apoptosis been ignored for so long? Perhaps, it is suggested, it is because we had difficulty in seeing that the integrity of the living whole could depend on the death of many of its components. Or perhaps it is simply because in Western societies the idea of death — even that of a cell — is repulsive, reminding us of our own mortality.

Rita Levi-Montalcini, winner of the Nobel prize for medicine in 1986, agrees that our concept of death at the cellular level has completely changed. “We used to look at death as only negative”, she says. “Today we also see it as positive.” She vividly recounts how she set up a laboratory in her bedroom in Turin following the purge of Jews from Italian universities in 1938. Studying chick embryos, she provided the first demonstration that nerve cells die if a certain signal — nerve growth factor — is removed. “It was wartime, with its battlefields and death”, she says. “These cells died, like soldiers at war. To me it looked like real life.”

“In me”, she goes on to say, “intuition prevails over reason — it is what science and art have in common.” Raff also talks about the relationship between the two cultures, lamenting the British disdain for science, particularly among politicians. “Not only do they not know about science”, he says, “but they do not want to know about science — they would consider it demeaning, somehow polluting their ethereal spirit. Isn't it depressing? It is depressing.”

Thankfully, there can be few happier marriages between the two cultures of scientific enquiry and the humanities than this enthralling documentary. It stimulates both curiosity and imagination; illuminates the world of cells with great charm and unpretentious artistry; and explains its biological wonders with poetic clarity. Leeuwenhoek would have been dying to see it. □

Peter Tallack is Book Review Editor of *Nature*.

Big blues

Robert Heller

Building IBM: Shaping an Industry and Its Technology. By Emerson W. Pugh. MIT Press: 1995. Pp. 405. \$29.95.

THE core of IBM's self-esteem has always been its technology. Emerson Pugh, after 35 years with IBM, understandably shares this whole-hearted admiration for its technical achievements — without, however, tackling critics who take a different view. They acknowledge, of course, the scientific riches that have poured from IBM laboratories, crowned by Nobel prizes and innumerable other distinctions. But the critics level one potent accusation: that too few of the great discoveries have surfaced in the company's products.

In other words, a significant lag existed between IBM's brains, so to speak, and its limbs. In so complex a product as a computer, so many devices and techniques are involved that the truth isn't easy to untangle. Nor is Pugh's course through the tangle easy to read or follow. But he touches on one clear, bizarre example of lag: IBM's discovery of reduced instruction set computing (RISC), primarily the work of John Cooke, an IBM fellow, which much enhances performance of smaller computers.

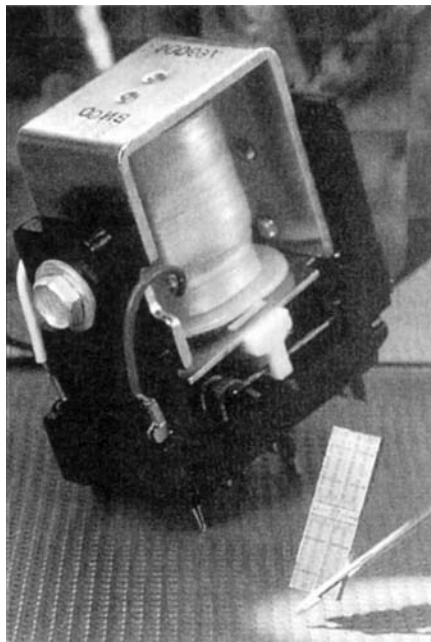
IBM, though, didn't use its own discovery in a workstation until 1990 — three years after Sun Microsystems and twice as long after the availability of RISC. How could such absurdity be allowed? Pugh explains: "RISC met considerable resistance in a company dedicated to extending the 360-370 architecture". The resistance was actually fierce: prosaic understatement is, alas, typical of an author who started in research and retired as an executive in 1992, deep in the thrilling rush of new technology to which RISC belongs.

Yet the book dwindles dully away with a mere three-paragraph account of the personal computer — the 1982 product that destroyed the old IBM. There's no mention of Intel and Microsoft, which in hardware and software seized vital leads from the potential champion. The resulting disasters caught this book in its toils. The project was originally financed by IBM, which withdrew during the agonizing drive to cut excessive costs and awful losses. The book's purpose, and its official-history character, however, remain unchanged.

It "tells how the technologies of IBM and its industry evolved from electro-mechanical devices and punched cards to integrated semiconductor circuits and magnetic disk storage devices of previously unimagined capabilities". Pugh's case is

that IBM dominated that evolution. It is true that its technologists, primarily engineers, contributed an endless stream of ingenious answers to the needs of users of punched-card machines and the computers that succeeded them — all the way to the development of the 360 machines.

Costing twice as much as the Manhattan Project, this range hit the industry with equivalent energy. Its birth traumas marked a watershed, not just in greatly speeding the spread of computing, but in IBM's whole research and development (R&D) activity. One omission sums up the weaknesses of both IBM's R&D and



Little and large: 1957 electromechanical switch and a modern 16-million-bit memory chip containing the equivalent of more than 20 million switches. Taken from *The Microprocessor: A Biography* by Michael S. Malone. Springer, \$29.95.

Pugh's book. He mentions Gene Amdahl, but ignores his genius. So did IBM. The company resisted his arguments for using fully integrated circuits in the 360: perhaps rightly, because the project's notorious delays might well have been longer still. But it then wrongly rejected Amdahl's plans for further developments using integrated chips.

The book ignores this crucial decision: it simply records Amdahl's departure to found his own company, one of several that proceeded to exploit IBM's growing fallibilities. These were initially most marked in large systems. The word STRETCH must be engraved on the corporate heart. Although the fastest computer of its day, the machine only achieved half the promised performance. The price of \$13.5 million had to be almost halved, and the company stopped taking orders. The outraged chairman, Tom Watson Jr, delivered so public and

scathing an attack on his engineers that work on big machines ground to a halt.

Pugh argues that the 360 range benefited from this failed pioneering — in "multiprogramming, memory protect, generalized interrupt, interleaving of memories, lookahead, the memory bus, a standard interface for input-output equipment, and the eight-bit character called the byte". But conservatism — of the kind that later caused resistance to RISC — was encouraged by the STRETCH failure and then by the massive convulsions required to complete the 360 project. Determined to preclude technological upheaval, IBM somehow lost sight of the elder Tom Watson's founding philosophy.

As Pugh rightly stresses, the elder Watson (which explains why he created the IBM fellowships) had technological vision as well as immense commercial acumen — and thus he didn't (as legend claims) need to be hauled protesting into the electronic era by his son. In 1932, Watson Sr said: "There is no business in the world which can hope to move forward if it does not keep abreast of the times, look into the future and study the probable demands of the future". Pugh (who irritatingly favours repetition) quotes this twice. But his book, while a workmanlike source on the early evolution of computery, misses the futuristic revolution that swept all before it — including the dominance of IBM. □

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Burt-watching

Chris Brand

Cyril Burt: Fraud or Framed? Edited by N. J. Mackintosh. Oxford University Press: 1995. Pp. 156. £19.99.

SIR Cyril Burt (1883–1971) thought that the broad heritability of IQ was high — yielding his notorious correlation of 0.77 between monozygotic twins reared apart (MZAs). Yet he estimated the narrow heritability as only 0.52: parents could pass on genetically to their children only a half of their IQ advantage (or disadvantage). Partly by this route, parents could transmit a third of their advantage (or disadvantage) in socioeconomic status. Countering regression to the mean, the class system would be refreshed by social mobility. Most of the brighter children would come from 'working class' homes and children's own IQs would account for some 50 per cent of eventual variance in socioeconomic status in their own generation. General intelligence (g), which Burt

had once found to relate to speed of intake of simple perceptual information, was frankly more important in life than were "personality traits"; but there were other sources of variation in mental abilities ("group factors"), and education should accord with children's ability profiles. There were also innate differences between races in *g*; but "they are small" in comparison with the big differences between individuals.

Burt's position was progressive enough to earn him a knighthood under a Labour government, but it infuriated rising social scientists and geneticists who abjured anything that could be linked to 'negative' eugenics. "Wouldn't it be great if it could be shown that Burt was really just an old fraud!" muttered one London educationist to Arthur Jensen in 1957. After Burt's death, closer scrutiny of his key work led at last to its being denounced as involving casualness or fraud, and bolder accusations of 'fascism' soon followed. Here, the eminent learning theorist Nicholas Mackintosh leads a hand-picked team of scholars in a reexamination of Burt's character and figurework.

Under-reporting of the details of what Burt himself often admitted to be "precarious" studies had always limited the scientific usefulness of Burt's research reports; and Burt's 0.77 figure is replaced today by the 0.78 estimate from the 43 MZA pairs studied in Minnesota. On the other hand, the charge of fraud against Burt has been rejected by most experts as 'not proven', chiefly because Burt's figures have crazily rough edges that no true fraud would have failed to smooth down — notably the 0.77 figure and also correlations for height and weight that remained the same across two big changes in sample size. Accepting all this, Mackintosh's contributors try to focus mainly on what only probably happened: "we are not trying a case in a court of law", they declare. As a verbal token, the term 'fabrication' is often used, rather than 'fraud'.

Thus liberated, the authors all find something quite interesting to say. Hans Eysenck colourfully exposes a level of deviousness in committee work that would put Burt well within the top 50 per cent of vice-chancellors and right up among the equally fabrication-prone Kepler, Newton and Freud. Steven Blinkhorn rehearses quite brilliantly, if a little self-indulgently, how Burt had every right to feel aggrieved at being written out of the history of factor analysis by Spearman and Thurstone. Nicholas Mascie-Taylor agrees to exonerate Burt from the best-known charge that his figures on social mobility were too perfect; but he details convincingly Burt's "deliberate deceptiveness" about his inadequate data on social mobility; and Jensen finds Burt a "brilliant eccentric" who was inexplicably and inexcusably "furtive". The book as a whole is fairly and indeed

beautifully written.

Such points, however, advance no very general claim about Burt. Eysenck, the giant of trait psychology, implicitly acknowledges the problem when he admits two quite different sides to Burt's character. Fortunately, Mackintosh, who writes three of the chapters, has more of an agenda and realizes the need to come off the fence — at least in his epilogue.

Taking Burt's 1966 MZA study first, Mackintosh establishes a magisterial authority by a sustained defence of the possibility that Burt had twin data from the 1920s. All that is missing from Mackintosh's account is any consideration of how, in 1966, Burt might have increased the number of MZA pairs on which relevant and supportive data were available. He could have embraced some of the better-separated pairs reported in J. Shields's *Monozygotic Twins* (1961). Hijacking of Shields's twins would explain Burt's otherwise peculiar statement that his 1966 paper would "bring together the evidence now available both from our own studies and from more recent investigators" and why he quite openly told Eysenck in 1971 that "our own studies" were mostly complete by 1939. It would also explain his wish, in 1969, while "calculating data on twins for Jencks", to have access to the library at University College London: simply, he needed the copious raw data in Shields's book from which he (or his assistant, Miss Conway) had once learned the crucial correlation of 0.77 that corresponded with his own. Plainly, at least something as 'devious' as this happened; but no-one knows what.

Having cleared Burt of fabrication of the twin data, however, Mackintosh maintains suspense for the volume with an engaging new line of attack. Perhaps the biggest difficulty for Burt-baiters is that Burt was spectacularly correct on so many points. How could he possibly have managed without data? So it is reasonable that an apparent error on Burt's part should arouse suspicion. As background to his 1969 figures (unsatisfactory, as usual) on declining educational standards in Britain from 1914, Burt explains that children's *g* levels had not changed. To demonstrate this he would have needed to test children in the 1960s with his 1914 tests; and he claims to have done that. But when this sort of exercise has been undertaken by others, as first in the 1940s, *g*-scoring has actually been found to be rising roughly by two IQ points a decade.

With this as the strongest case that Mackintosh finds he can make for fabrication, many would-be accusers will hold their peace. After all, the secular IQ rise is not found for Burt's tests but chiefly for others where important scoring opportunities can be gained by guessing or skipping harder items instead of persevering; Burt plainly resisted the temptation to

recognize an IQ-type rise that would helpfully have thrown supposed declining educational standards into sharper relief; and even James Flynn, the chief exponent of the rise, does not himself believe it to have been in true intelligence — only in whatever conventional IQ tests measure. So Burt actually notches up yet another success — at least while agreeing with Flynn is Mackintosh's touchstone. Eysenck and Jensen themselves have admittedly interpreted Flynn's work as indicating a substantial *g* rise, so Mackintosh might squeeze more testimony to Burt's psychopathy from them. Properly considered, however, Mackintosh's academic whodunit marks a further step towards Burt's rehabilitation. □

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Children's Books

Nature plans to publish on 16 November a supplement in which children's books and software will be reviewed. The supplement will cover new publications for children of all ages.

Publishers are invited to send suitable material for consideration, taking note of the following criteria:

- Only books and software issued in 1995 will be considered;
- Books and software dealing with any aspect of science, technology, medicine, natural history or the environment are eligible (including encyclopaedias, dictionaries and games), although school curricula texts are excluded;
- The main language used must be English;
- If possible, cross-platform software (both Macintosh and PC) should be provided.

Publications for review should be sent immediately, together with details of price and availability, to Peter Tallack, Book Review Editor, *Nature*, 4 Crinan Street, London N1 9XW, UK (tel: +44 (0)171 843 4567; fax: +44 (0)171 843 4596/7; e-mail: p.tallack@nature.com).

New in paperback

Black Holes and The Universe by Igor Novikov. Cambridge University Press, £5.95.

Planetary Overload: Global Environmental Change and the Health of the Human Species by A. J. McMichael. Cambridge University Press, £7.95.

All Was Light: An Introduction to Newton's Opticks by A. Rupert Hall. Oxford University Press, £12.95.

Out of the Blue: Depression and Human Nature by David B. Cohen. Norton, \$13.95, £9.95.

Hasty basics

Steven H. Strogatz

Introduction to Nonlinear Science. By G. Nicolis. Cambridge University Press: 1995. Pp. 254. £35, \$59.95 (hbk); £13.95, \$24.95 (pbk).

NONLINEAR dynamics was a hot subject in the early 1980s. I remember the excitement and frustration of our informal seminars where a few graduate students and young professors struggled to explain unfamiliar things to one another, the blind leading the blind. We prayed for a lucid textbook.

Many books have been written since then, some mathematically rigorous, others geared more towards the physical sciences. But in the preface to his graduate text *Introduction to Nonlinear Science*, G. Nicolis argues that most of these books take too narrow a view of nonlinear dynamics — they ignore spatially extended systems as well as the statistical aspects of the subject. Nicolis tries admirably to present a coherent account of nonlinear science in a way that does justice to all its facets. Unfortunately he has not quite succeeded. In his eagerness to cover topics long neglected, he skips too many of the fundamentals.

Two examples recur throughout: thermal convection in a layer of fluid heated

from below, and reaction-diffusion models of chemical oscillations and patterns. After deriving the governing differential equations, Nicolis develops tools to analyse them. First come the techniques for low-dimensional systems: phase space, linear stability analysis and local bifurcation theory. The treatment is perfunctory, except for the derivation of normal forms, handled nicely using the method of multiple scales and solvability conditions.

The same approach reappears in the more complicated setting of spatially extended systems, the strongest part of the book. Here Nicolis derives the complex Ginzburg-Landau equation and other universal models for pattern formation. He also gives clear explanations for Turing patterns in chemical systems and the various instabilities in the convection problem, for both small and large aspect ratios. A final chapter on chaos again lapses into superficiality when discussing standard topics (period doubling, circle maps and intermittency), then recovers in its treatment of ergodicity, mixing and other probabilistic notions.

The book is aimed at graduate students, but, given its hasty treatment of the basics, I would recommend it only as a supplement, rather than as a primary text. □

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Glimpses of fundamental harmony

S. S. Schweber

The Quantum Theory of Fields. Volume 1: Foundations. By Steven Weinberg. Cambridge University Press: 1995. Pp. 609. £35, \$49.95.

ROBERT Oppenheimer once pointed out that all great scientific advances have two traits. On the one hand, they provide know-how, an enrichment of technique, enabling us to do what we could not do before, or to do it better; and, on the other hand, they contribute knowledge, an answer and reformulation of questions that have long excited man's curiosity, something to contemplate, "a glimpse of harmony and order, a thing of beauty".

The quantum theory of fields is no exception. It allows precise calculation of the scattering processes arising from the interaction of the electroweak and strong forces (up to energies of the order of 10^{12} electronvolts) and the theoretical calculations agree with empirical data to a remarkable degree. And the theory has given us a glimpse of the harmony and order of the subnuclear world.

Quantum field theory was developed during the late 1920s as a general framework to describe in quantum mechanical terms the interaction of charged particles with the electromagnetic field. During the 1930s, the framework was extended by Enrico Fermi to model beta-decay and by Hideki Yukawa to explain nuclear forces. All these field theories have two features in common: they synthesize quantum mechanics and special relativity, and the interaction between the fields is at a single point in space-time. Local quantum field theories are flawed, however: their perturbative solutions are divergent, the infinities encountered resulting from the locality of the interaction.

After the Second World War, stimulated by the reliable and precise measurements by Willis Lamb and by Isidor Rabi of the ground-state structure of hydrogen, renormalization theory was formulated to circumvent the divergence difficulties with higher-order calculations in quantum electrodynamics. Freeman Dyson showed that a 'renormalization' of the charge and mass parameters (and a

rescaling of the field operators) of the Lagrangian function that describes the theory could absorb all the divergences in quantum electrodynamics. More generally, Dyson demonstrated that only certain kinds of quantum field theories can absorb *all* the infinities by a redefinition of a *finite* number of parameters. He called such theories 'renormalizable'. Renormalizability immediately became a criterion for theory selection and played an important part in the development of the standard model of the electroweak and strong interactions.

During the past 20 years, our understanding of quantum field theories and of renormalization has changed dramatically. The work of Kenneth Wilson and Steven Weinberg has given us a more limited view: all existing theoretical representations of phenomena are only partial descriptions that depend on the energy at which the interactions are analysed. Successful quantum field theories are in fact low-energy approximations to a more fundamental theory that may or may not be a field theory. Weinberg and others have shown that the reason quantum field theory describes physics at accessible energies "is that any relativistic quantum theory will at sufficiently low energies look like a quantum field theory".

Weinberg, one of the chief architects of the standard model and a major contributor to the development of quantum field theory since the 1960s, provides an impressively lucid and thorough presentation of the subject from this modern viewpoint. He believes that physics describes observable phenomena and that all physical measurements and interactions can be considered as scattering processes; and in this context he shows that quantum field theory is essentially the only way of reconciling the principles of quantum mechanics with those of the special theory of relativity. The first volume of *The Quantum Theory of Fields* covers the foundations of quantum field theory and quantum electrodynamics, whereas the second volume, due to be published next year, will deal with the important advances of the past two decades, such as non-Abelian gauge theories, broken symmetries, the renormalization group and anomalies.

Weinberg manages to present difficult topics with richness of meaning and marvellous clarity. Full of valuable insights, his treatise is sure to become a classic, doing for quantum field theory what Dirac's *Quantum Mechanics* did for quantum mechanics. I eagerly await the publication of the second volume. □

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Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor

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Thyroid-hormone and retinoic-acid receptors exert their regulatory functions by acting as both activators and repressors of gene expression. A nuclear receptor co-repressor (N-CoR) of relative molecular mass 270K has been identified which mediates ligand-independent inhibition of gene transcription by these receptors, suggesting that the molecular mechanisms of repression by thyroid-hormone and retinoic-acid receptors are analogous to the co-repressor-dependent transcriptional inhibitory mechanisms of yeast and *Drosophila*.

THYROID-hormone and retinoic-acid receptors belong to the nuclear receptor family of ligand-regulated transcription factors and modulate specific developmental and homeostatic programmes of gene expression by binding to *cis*-acting DNA sequences in target genes¹. These receptors bind as heterodimers with the retinoid-X receptor (RXR)²⁻⁶ to cognate DNA sites that are generally organized as direct repeats of a core binding motif, in a polarity-specific fashion. As a consequence of binding to these sites, thyroid-hormone and retinoic-acid receptors (RAR) can exert both positive and negative control of gene transcription.

The thyroid hormone receptor (T₃R) was originally identified on the basis of its homology to the viral oncogene *erbA* of the avian erythroblastosis virus (AEV)⁷⁻⁹. v-ErbA, which in contrast to the wild-type receptor does not bind thyroid hormone with high affinity, functions as a constitutive transcriptional repressor of both thyroid-hormone- and retinoic-acid-responsive genes^{8,10-16}. The cellular thyroid-hormone receptor was also found to repress transcription of target genes in the absence of ligand^{11-14,17,18}. In most cases, ligand-independent repression appears to result from an active, transferable repression function within the ligand binding domain^{19,21}. Fusion of the C-terminal domains of v-ErbA, T₃R and RAR, but not RXR²², to the DNA-binding domain of GAL4 generated GAL4-site-dependent transcriptional repressors²¹.

The molecular mechanisms responsible for nuclear receptor transcriptional silencing are not well understood, but have been suspected to involve interaction with basal transcription factors, including TFIIB²³⁻²⁶. *In vitro* unliganded thyroid-hormone receptor can inhibit transcription by preventing formation of the preinitiation complex²⁵. Further data, however, indicate that the distal thyroid-hormone-receptor C-terminal regions that interact with TFIIB are not sufficient to confer repression in cells. Indeed, the regions in the hinge and N-terminal part of the ligand-binding domain of the thyroid hormone receptor are required for silencing^{24,27,28} and imply the existence of additional interacting factors that are required for ligand-independent repression^{28,29}.

We now report the identification of a 270K protein that associates with DNA-bound thyroid-hormone/retinoid-X-receptor heterodimers in the absence of thyroid hormone, but not in its presence. Isolation of the complementary DNA encoding this factor has revealed a co-repressor containing a nuclear receptor interaction domain in the distal C terminus, and a transferable

repressor domain in the N terminus. This factor interacts only with thyroid-hormone and retinoic-acid receptors. Specific mutations in the hinge region of thyroid hormone receptor that abolish interactions with the 270K protein coordinately eliminated the ligand-independent repression function of the thyroid hormone receptor. Together, these data suggest that this 270K protein, referred to as N-CoR for nuclear receptor co-repressor, mediates ligand-independent transcriptional repression.

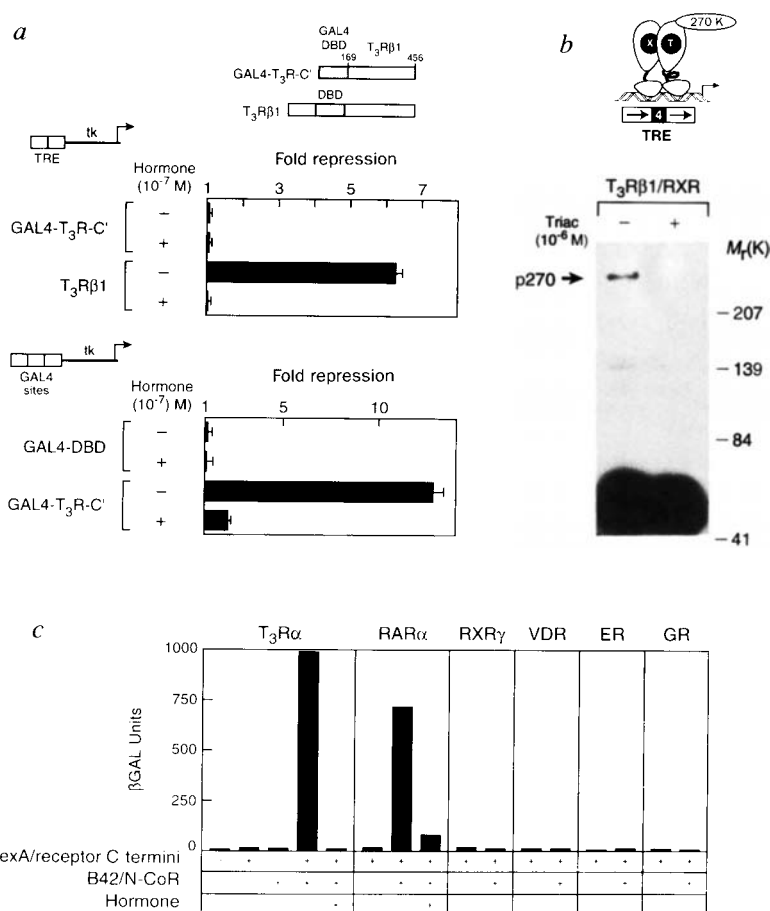
Identification of p270/N-CoR

As previously described, the C terminus of the thyroid-hormone receptor $\beta 1$ (T₃R $\beta 1$) transferred active repression when fused to the GAL4 DNA-binding domain. Thus the critical information required for repression resides in the ligand-binding domain (see Fig. 1a for example). To investigate the mechanism by which the thyroid hormone receptor can repress gene expression, we evaluated the ability of specific factor(s) to associate with the thyroid hormone receptor when bound as a heterodimer to its cognate DNA site. A biotinylated thyroid-hormone receptor DNA-response element was used to assemble stable thyroid-hormone receptor/retinoid-X receptor (T₃R/RXR) heterodimers on a streptavidin agarose matrix. Whole-cell extracts prepared from CV-1 cells were then added, incubated, washed, and bound material separated by SDS polyacrylamide gel electrophoresis. After transfer to nitrocellulose membranes, thyroid-hormone-receptor-associated proteins were identified using ³²P-labelled thyroid-hormone-receptor C terminus. This binding assay revealed that a 270K protein (p270) was capable of selectively binding to the unliganded T₃R/RXR heterodimer (Fig. 1c). Addition of ligand released p270 (Fig. 1c) and induced interaction with the previously identified 140K and 160K proteins^{30,32}.

cDNA encoding the p270 factor

To isolate the cDNA encoding p270, we used the yeast two-hybrid screen^{33,34}. Seven of the identified clones interacted only in the absence of TRIAC (3,3',5-triiodothyroacetic acid) and were further evaluated. Of these seven clones, only one encoded a messenger RNA of sufficient length to encode a 270K protein, and this clone interacted more strongly than the other six. Using the cDNA recovered from this clone, a mouse pituitary cDNA library was screened, until the entire coding sequence was obtained and sequenced. The murine cDNA encompassed a

FIG. 1 A 270K factor interacts with unliganded T_3 receptor heterodimers on DNA. **a**, Luciferase transcription units under control of a promoter, containing either two copies of a synthetic DR+4 element of GAL4-binding sites (17-mer) were used for co-transfection assays in CV1 cells with a CMV transcription unit expressing a GAL4/ T_3 R β 1 (amino acids 165–456) fusion protein or a T_3 R β 1 transcription unit under control of the RSV promoter. **b**, Identification of a 270K protein that interacts with T_3 R/RXR heterodimers on a DR+4 site. Purified T_3 R β 1 and RXR- α bound biotinylated DR+4 oligonucleotide. Streptavidin matrix was incubated with CV1 whole-cell extract in the presence or absence of 10^{-7} M TRIAC (3,3',5-triiodothyroacetic acid). Proteins were detected using unliganded 32 P-GST- T_3 R β 1 carboxy terminus probe. The strongest signal at 52K–54K represents binding of the probe to RXR and T_3 receptors. The only detectable protein differentially bound in a ligand-independent fashion was a 270K protein (arrow). **c**, Characterization of a novel protein that interacts with thyroid-hormone and retinoic-acid receptors in a hormone-independent manner identified in the yeast two-hybrid screen. The identified interacting protein was expressed as a fusion protein with the B42 activation domain. The following baits were tested: LexA- T_3 R α 1_(122–410), LexA-RAR α _(143–462), LexA-RXR γ _(134–463), LexA-VDR_(88–427), LexA-ER_(251–595) and LexA-GR_(465–795). β -Galactosidase activity was determined for yeast (*S. cerevisiae*, EGY48)³⁴ containing the reporter plasmid (pSH18–34), the prey, and the different bait constructs. Hormones were 10^{-7} M TRIAC (first panel) and 10^{-7} M 9-cis retinoic acid (second panel). **METHODS.** **a**, Co-transfections were performed using CMV/GAL4/ T_3 R β 1_(165–462) fusion protein, RSV/ T_3 R β 1, DR+4-luciferase, and GAL4-tk-luciferase as transcription factor and reporter plasmids, as previously described⁷. **b**, Oligonucleotides for the DR+4 site (sense strand: 5'-biotTCTGGAGGTGACAGGAGGACAGCCC-3') were synthesized to contain biotin residues at the 5' end using biotin phosphoramidite. Binding was performed using 1 μ g double-stranded oligonucleotide and 100 ng of h T_3 R β and hRXR- α . Receptor-DNA complexes were purified using streptavidin-agarose, washed and incubated with or without 10^{-7} M TRIAC. CV1 whole-cell extracts (1.5 μ g per lane), were incubated, washed and bound proteins were resolved by electrophoresis. The T_3 R β 1 (amino acids 13–456) was cloned into the vector pGEX-2TK, phosphorylated using [γ - 32 P]ATP, and used to detect the proteins. **c**, The yeast (*S. cerevisiae*) strain EGY48 (ref. 40) was transformed with 'bait vector'



(pEG202- T_3 R α _(122–410)), the lacZ reporter plasmid pSH18–34, and a HeLa oligo(dT)-primed fusion library³⁴. 3.5×10^7 yeast cells were plated out on -Ura-His-Trp-Leu medium containing 2% galactose. Of the colonies obtained, 400 were replica-plated on -Ura-His-Trp galactose medium containing 0.1% X-gal. To test the affinity nuclear receptors, yeast clones containing reporter, the different nuclear receptor baits and the prey were assayed for β -galactosidase activity.

and retinoic-acid-receptor C terminus (Fig. 3a, b, d and data not shown). Similar ligand-independent association was observed with v-ErBA (data not shown). In contrast, when they were bound to DNA, both thyroid-hormone- and retinoic-acid-receptor heterodimers exhibited ligand-dependent release of the 270K holoprotein (Fig. 1b and ref. 32). These data indicate that the configuration of the heterodimer on DNA imposes an allosteric effect which is a critical aspect of the ligand-dependent release of the 270K protein. Because the 270K protein fails to associate with retinoid-X receptors, it is likely to be bound exclusively to the thyroid-hormone-receptor component of the DNA-associated heterodimer pair.

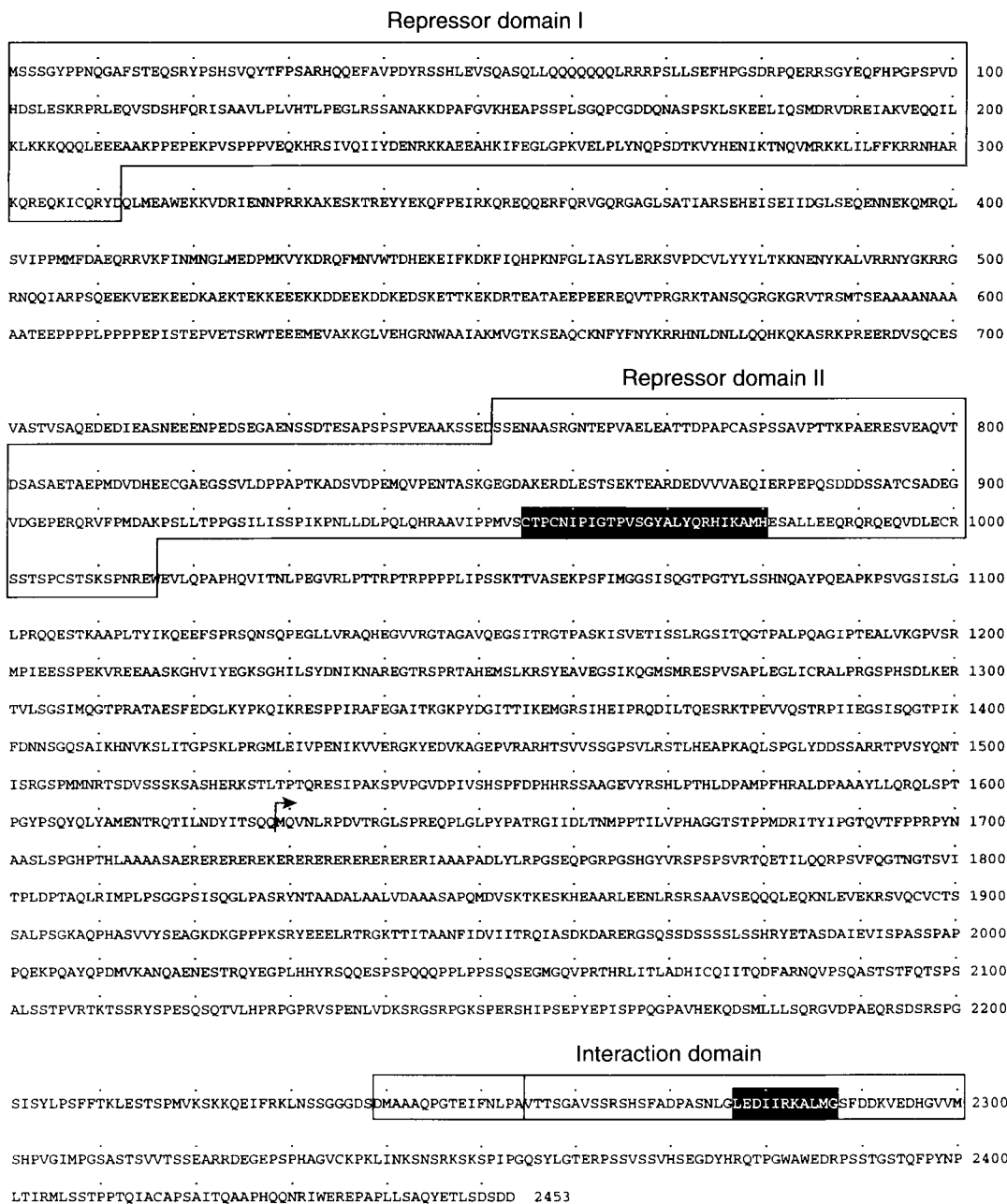
To confirm that N-CoR corresponds to the 270K interacting protein identified biochemically (Fig. 1b), a guinea-pig antiserum was generated against amino acids 811–996 of the recombinant protein. This antiserum specifically recognized the 270K protein purified from L-cell extracts using GST- T_3 R β 1 (Fig. 3b). Furthermore, the 270K protein that interacted with GST- T_3 R β 1 was detected using 32 P-GST-RAR α (Fig. 3b). Together these data indicate that the cDNA clone encodes the p270 protein. Furthermore, immunodepletion of whole-cell extracts with anti-N-CoR antiserum results in almost quantitative removal of p270 (data not shown).

Transfection of COS cells with a vector containing a transcription unit expressing N-CoR with a C-terminal Flag epitope permitted histochemical localization of the protein to the nucleus

7,359-nucleotide open reading frame that predicted a 2,453-amino-acid protein (Fig. 2). An alternatively spliced variant, which results in a loss of amino acids 2,333–2,371, was also detected. A partial sequence of this protein (amino acids 1,792–2,453) has been reported to GenBank and is referred to as RIP13 by Seol *et al.*³⁵. No significant homology with any other protein in the available data bases was detected, but a potential Cys-Cys, His-His zinc-finger motif was noted at amino acids 965–980. The specificity of interaction with the T_3 receptor C terminus was evaluated by examining interactions with other nuclear receptors, both in yeast and *in vitro*. The results of these analyses are shown in Figs 1c and 3. In addition to strong interactions, as recorded by the yeast two-hybrid assay with the T_3 receptor C terminus, the factor also interacted with the homologous region of the retinoic-acid receptor- α (RAR- α). In both cases, interactions were abolished upon the addition of ligand. In contrast, no evidence of interactions was detected with the C termini of the oestrogen, glucocorticoid, vitamin D or retinoid-X receptors. Direct binding was initially confirmed by interaction assay with glutathione-S-transferase (GST) fusion proteins, using the *in vitro* translated 724-amino-acid C-terminal region of N-CoR, which revealed strong interactions with only the unliganded thyroid-hormone- and retinoic-acid-receptor C termini, and no or minimal interactions with the retinoid-X-receptor C terminus (Fig. 3a). But when full-length N-CoR was used, ligand had little or no effect on its interaction with the thyroid-hormone-

Fig. 2 Amino-acid sequence of N-CoR, as predicted by the encoding murine cDNA. The double-stranded sequence of a series of highly overlapping cDNA insets, identified by four successive rounds of screening a murine pituitary cDNA library⁵¹, predict an open reading frame encoding a 2,453-amino-acid protein. The sequence was submitted to GenBank (accession number, U35312). The sequence of the interaction domain located in the C terminus is shown by the boxed region, corresponding to amino acids 2,255–2,300. The Chou–Fassman and Garnier–Osguthorpe–Robson algorithms predict an α -helix, shown in the shaded box. The position of transferable repression domain (Fig. 6) are indicated. A putative zinc-finger motif is indicated in repression domain II. The arrow at amino acid 1,628 shows the initiator codon in use to express the C-terminal fragment, referred to as N-CoR-C' (amino acids 1,628–2,453) *in vitro*. A partial sequence of this protein (amino acids 1,792–2,453) was found in GenBank (accession number, U220160).

METHODS. The 900-bp insert recovered from the initial screen in yeast was used to screen a murine anterior pituitary cDNA library. Four successive rounds of screening were then used to isolate multiple cDNA inserts, all of which were subjected to complete double-stranded sequencing by the enzymatic procedures; considerable overlaps confirmed the assignment of the ORF and the *in vitro* transcription/translation in reticulocyte lysate generated a 270K protein, confirming the ORF.



(Fig. 3c). Antiserum against the N terminus of T₃Rβ1 was used to immunoprecipitate the thyroid hormone receptor and any associated proteins from whole-cell extracts of COS cells expressing the N-CoR-Flag protein and T₃Rβ1. As shown in Fig. 3d, the 270K factor was specifically immunoprecipitated in a thyroid-hormone-receptor-dependent fashion; therefore, the interaction between N-CoR and the thyroid hormone receptor occurs in the cell. Addition of T₃ or TRIAC, even at 10⁻⁶ M, failed to disrupt the association between p270 and the thyroid hormone receptor (Fig. 3b), consistent with the results obtained using N-CoR holoprotein in GST interaction assays. The gene encoding N-CoR appears to be widely expressed, but at significantly different levels, in various tissues and cell lines (Fig. 3e).

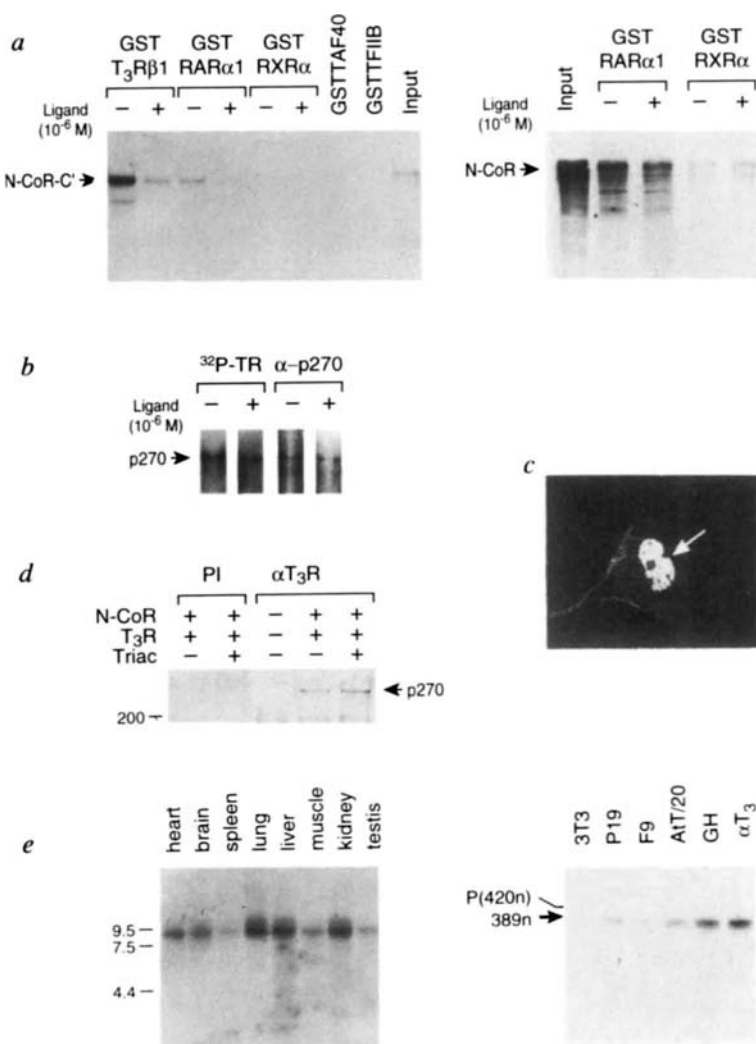
N-CoR interacts with T₃R hinge region

Using a series of N- and C-terminally truncated proteins, the interaction domain of N-CoR was localized to a 60-amino-acid region in the C terminus (Fig. 4a). A further N-terminal deletion in the human homologue (data not shown) suggested that the

critical region is encompassed by a 45-amino-acid sequence (residues 2,255–2,300). The algorithms described by Chou–Fassman and Garnier–Osguthorpe–Robson predict an α -helical region in the C terminus of this interaction domain (Fig. 2). To identify the sequences in the thyroid hormone receptor that are required for interaction with N-CoR, we expressed the C-terminal 397 amino acids of N-CoR fused to GST and tested for interaction with N- and C-terminal-deletion mutants of T₃Rβ1 (Fig. 4b d). These experiments revealed the hinge region (amino acids 203–230) as the critical binding domain, but with additional contributions from the N-terminal portion of the ligand-binding domain (amino acids 230–260). Comparison of the sequence of the thyroid-hormone and retinoic-acid receptors, which interact with the 270K protein, and of other nuclear receptors that do not interact (Fig. 5a), revealed a region that shows significant homology only between the two nuclear receptors that showed ligand-independent interaction with N-CoR, as well as v-ErbA and the vitamin D receptor. Based on the homology, a series of mutations was generated in the hinge, substituting conserved

FIG. 3 Biochemical characterization of N-CoR interactions. **a**, Biochemical demonstration of N-CoR interaction with T₃R and RAR. The ability of N-CoR-C' (amino acids 1,628–2,453) (left panel) or holoprotein (right panel), to bind to bacterially expressed glutathione-S-transferase (GST) fusion proteins of full-length thyroid-hormone, retinoic-acid and retinoid-X receptors^{2,51} was investigated in the presence and absence of ligand: TRIAC (10⁻⁶ M), all-*trans* retinoic acid (10⁻⁶ M), and 9-*cis*-retinoic acid (10⁻⁶ M), respectively. **b**, The protein encoded by the N-CoR cDNA corresponds to the p270 that binds to T₃R/RXR heterodimers on DNA. GST-T₃R-C' fusion proteins were used to bind whole L-cell extracts, and bound proteins were analysed using either ³²P-TR C-terminal probe or anti-N-CoR sera (α-p270). Both assays identified the same 270K protein. –/+, Binding in the presence (+) or absence (–) of 10⁻⁶ M T₃. **c**, N-CoR is a nuclear protein. The cDNA encoding N-CoR protein, containing an in-frame C-terminal 14-amino-acid extension, ([EYKEEEK]₂: 'flag' epitope) was generated and expressed in COS cells. Use of the anti-Flag M2 monoclonal antibodies permitted detection of the N-CoR protein in the nucleus, but not cytoplasm, of transfected cells. **d**, Immunoprecipitation of thyroid-hormone receptor complexes with a TR antibody (αT₃R) results in coimmunoprecipitation of N-CoR, detected after western blotting using anti-Flag M2 antibodies. Preimmune serum (PI) served as negative control. Experiments were performed in the presence or absence of 10⁻⁶ or 10⁻⁷ M TRIAC. **e**, Poly(A)-selected RNA from a series of murine tissues was fractionated on denaturing gels, transferred to membranes, and hybridized against a N-CoR-specified cDNA probe (left panel). An mRNA species migrating ~9.2 kb was detected. RNase protection assays were conducted with various tissues and cell lines, a few of which are shown in the right panel. N-CoR transcripts were detected in every cell line and tissue analysed.

METHODS. **a**, GST fusion proteins of hRARα, hT₃Rβ, and hRXRα were prepared and purified on glutathione agarose. ³⁵S-labelled full-length N-CoR or N-CoR C' was prepared by *in vitro* transcription/translation. These ³⁵S-methionine-labelled proteins were incubated with 2–3 μg GST fusion protein bound to 25 μl glutathione-agarose beads, washed, eluted and fractionated by SDS-polyacrylamide gel electrophoresis; 10% input is shown. **b**, For western blot analysis of p270, the nitrocellulose membrane blotted after SDS-PAGE of eluted GST-T₃R bound proteins from L-cell or CV1-cell extract was incubated with a guinea-pig polyclonal antiserum raised against N-CoR at a 1:500 dilution; detection was performed using an ECL western blotting system (Amersham). p270 bound to T₃R/RXR heterodimers was detected using ³²P-labelled T₃R C terminus. **c**, **d**, Two oligonucleotides corresponding to two copies of the Flag sequence, including a BamHI site (5') and a NotI site (3') for cloning, were synthesized (sense: 5'-GCGGGATCCGACTACAAGGACGACGATGACAAGGACTACAAGGACGACGATGACAAGTGAGCGGCCGCGGCCAGAG-3'), annealed digested with BamHI and NotI, fused in frame at the C terminus of N-CoR, and placed under the control of a CMV transcription unit. COS cells were transfected and used for immunohistochemical analysis using the



IBI Flag monoclonal antibody and fluorescent secondary antibody. **e**, Poly(A)⁺ RNA from adult mouse tissues (2 μg per lane) was subjected to denaturing gel electrophoresis, transferred to a nylon membrane, and hybridized to a ³²P-labelled DNA probe corresponding to amino acids 791–1,147 of N-CoR. The RNase protection used a 389-nucleotide fragment corresponding to amino acid 1,715–1,844 which was cloned in 3' to 5' direction into pSP65. A 420-nucleotide ³²P-labelled SP6 RNA transcript was generated. The control for proportionate loading was provided using α, β-actin probes.

residues with amino acids that might alter the helical properties of the region. Interactions with p270 were evaluated in the context of the T₃Rβ1 holoprotein, or the receptor C terminus (Fig. 5b). Mutated proteins were initially evaluated for maintaining normal heterodimeric DNA-binding properties. Mutations T₃RβE215R and of residues AHT at positions 223,224,227→GGA (T₃Rβ-AHT_M) were selected for more extensive analysis based on the observation that DNA binding was entirely comparable to the wild-type receptor (Fig. 5c). They were then evaluated for their ability to bind the N-CoR C terminus using GST fusion proteins with the receptor C terminus. Binding of T₃RβE215R to N-CoR C terminus was equivalent to the wild-type receptor. However, T₃Rβ-AHT_M was quite ineffective in binding ³⁵S-labelled N-CoR C terminus (Fig. 5d), or holo N-CoR (data not shown).

p270 as co-repressor of T₃R and RAR

Based on these results, the wild-type receptor, T₃RβE215R and T₃Rβ-AHT_M were tested for their capacity to mediate ligand-

independent repression. As shown in Fig. 6a, T₃RβE215R can fully inhibit transcription, whereas T₃Rβ-AHT_M was incapable of repression. To confirm the specificity of these data, variant and wild-type receptors were evaluated for their ability to function as activators; we found that T₃-dependent activation was actually somewhat higher with T₃Rβ-AHT_M than with the wild-type receptor (Fig. 6a).

Based on these observations, we postulated that N-CoR functions as a critical co-repressor of the thyroid-hormone and retinoic-acid receptors, because it was detected stably bound to DNA-associated thyroid-hormone receptor in the absence of ligand, and receptor mutations that eliminate the binding to the 270K protein simultaneously abolish ligand-independent transcriptional repression. If this model were correct, N-CoR would be capable of conferring repression itself if fused to a heterologous DNA-binding domain. To do this experiment, the GAL4 DNA-binding domain was fused to N-CoR, replacing the C-terminal interaction domain to avoid unwanted DNA-dependent nuclear receptor interaction, and its ability to act as

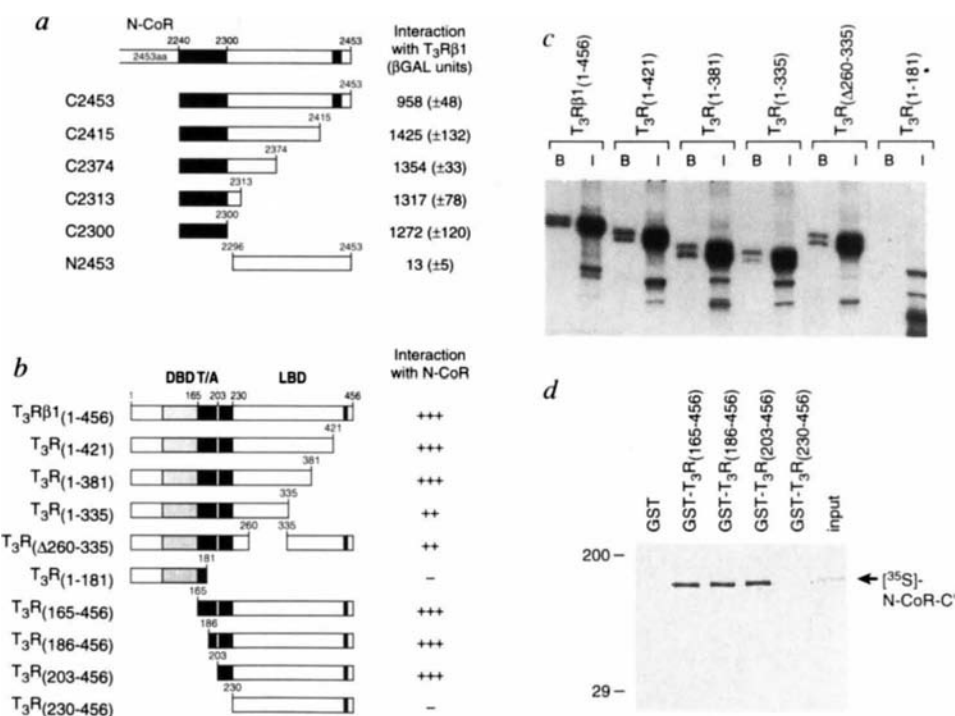


FIG. 4 Mapping of the N-CoR interaction domain to a conserved region in the hinge of T₃R and RAR. **a**, Mapping of the interaction domain of N-CoR. Using PCR techniques, a series of five different C-terminal and one N-terminal deletion of the N-CoR C terminus (amino acids 2,240–2,453) were cloned into pJG4-5. Interactions were studied using LexA-T₃Rα1_(122–410) as a bait. β-Galactosidase was assayed from yeast containing bait, reporter plasmid pSH18-34, and the different prey constructs. **b**, Summary of results of N-CoR binding to a series of T₃-receptor N'- and C'-terminal deletion proteins, determined using either GST-N-CoR C terminus, GST-T₃Rα1, or GST-T₃Rβ1 fusion proteins and ³⁵S-methionine-labelled T₃Rβ or N-CoR C-terminus proteins in a GST co-precipitation assay (Fig. 2). T₃Rβ deletions to amino acid 230 or beyond amino acid 260 failed to bind N-CoR. **c**, GST interaction assays of TNT translated ³⁵S-methionine labelled T₃Rβ1 variants. GST pull-down assays were performed with a fusion of GST and the C terminus (amino acid 2,057–2,453) of N-CoR as described above. Bound (B),

input (I), and (10%) of the different ³⁵S-labelled proteins are indicated. **d**, GST interaction assays of TNT translated ³⁵S-methionine labelled p270 C terminus. These assays were performed with different bacterially expressed GST-T₃Rβ1 fusion proteins (GST-T₃Rβ1_(165–456), GST-T₃Rβ1_(186–456), GST-T₃Rβ1_(203–456), and GST-T₃Rβ1_(230–456)) using ³⁵S-methionine labelled p270 C terminus (amino acid 1,629–2,453), as described above. Input, 10%.

METHODS. **c, d**, Thyroid-hormone receptor mutants were generated using appropriate synthetic oligonucleotides to synthesize PCR fragments or naturally occurring restriction sites in the hT₃Rβ cDNA were used to generate cDNAs encoding five different C-terminal translated and internal variants of T₃Rβ1. All constructs were subjected to DNA sequencing and used to generate cDNAs encoding GST fusion proteins or directly cloned into pBKS inserts for TNT reactions. In each case, binding was assessed by GST interaction assays.

a repressor was evaluated. These experiments revealed that N-CoR, on binding DNA, mediated a 15- to 25-fold repression, depending on promoter context and cell type (Fig. 6b). Similarly, repression was obtained on fusion of N-CoR holoprotein C-terminal of the GAL4 DNA-binding domain (data not shown). We therefore refer to this protein as the nuclear receptor co-repressor, N-CoR, and the region to which it binds in T₃R and RAR as the CoR box. To assess which regions of N-CoR might mediate the repression activities, a series of 300–400-amino-acid fragments, spanning the entire protein, was used to generate GAL4 fusion proteins. As shown in Fig. 6b, the extreme N-terminal fragment consistently transferred a high level (7–15-fold) of repression in the cell types evaluated. The N-terminal region contains both glutamine-rich regions and basic amino-acid residues, both implicated in repressor domains of other proteins^{36–38}. A smaller repression function could be transferred by a second region (residues 752–1,116), which encompasses a potential zinc-finger motif. None of the fragments conferred significant activation of transcription.

Discussion

In addition to their well established roles in ligand-dependent activation, nuclear receptors can function as active repressors^{8,13,19,21}. Transcriptional repression by thyroid-hor-

mone and retinoic-acid receptors occurs in the absence of ligand when they are bound as heterodimers with retinoid-X receptor to their cognate DNA sites¹⁸. Previous studies have suggested the existence of a co-repressor(s) required for ligand-independent silencing functions of the thyroid-hormone and retinoic-acid receptors^{28,29,39}. Our data suggest that N-CoR, a 270K nuclear protein that selectively interacts with DNA-bound T₃R/RXR or RAR/RXR heterodimers, and which is released from DNA-associated heterodimers upon binding of ligand, serves as such a co-repressor. Because redundancy of critical regulatory functions is commonly observed in biology, additional co-repressors may exist. We note, however, that N-CoR is the dominant protein to interact with the thyroid-hormone and retinoic-acid receptors in biochemical assays in all cells we examined. The nuclear receptor interaction domain and major repression domains are located in the extreme C- and N-terminal regions, respectively. The observations that N-CoR can itself transfer active repression to a heterologous DNA-binding domain implies that repression by the thyroid hormone receptor does not necessarily require interaction with TFIIB through the distal C terminus. Conversely, we note that mutations in the hinge domain of the thyroid hormone receptor that entirely abolish interaction with N-CoR and ligand-independent repression do not reduce interactions with TFIIB (data not shown). These data

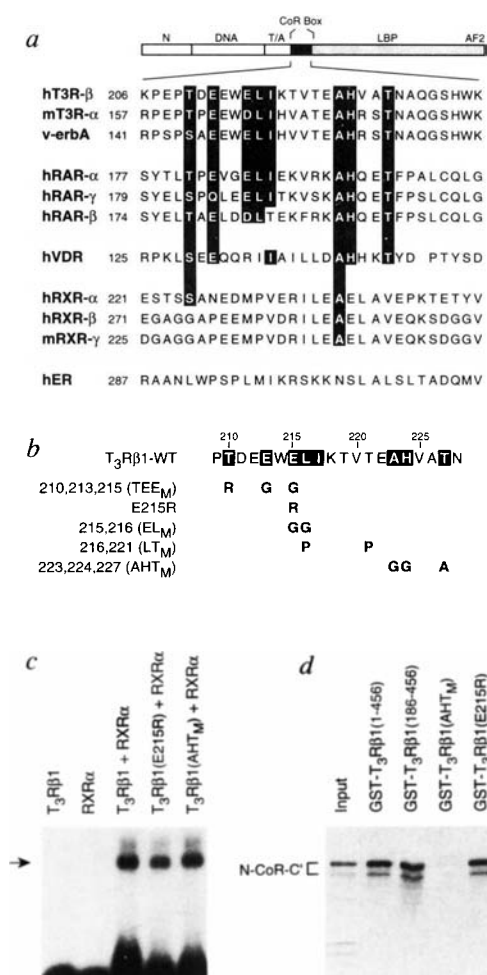


FIG. 5 N-CoR binding is required for repression actions of the T₃ receptor. **a**, Alignment of the hinge region of T₃Rs and RARs reveals a highly conserved region within this N-CoR binding domain, referred to as the CoR box. Residues conserved between RAR and T₃R are highlighted. **b**, A series of mutations were generated using synthetic oligonucleotides and site-directed mutagenesis of T₃Rβ1, as indicated. Each mutant was sequenced and expressed after placement in pBKS. **c**, ³⁵S-methionine labelled proteins generated by *in vitro* transcription/translation (TNT) of these mutant T₃Rβ1 cDNAs were analysed on SDS/PAGE and used to perform gel retardation assays. Wild-type and mutant T₃Rβ1 and RXRα proteins generated by *in vitro* transcription/translation were used in mobility shift assays using a ³²P-labelled DR + 4 site. The heterodimeric complex bound to DNA is indicated with an arrow. Only two mutations were virtually equivalent to wild type in binding activity (E215→R and AHT_M). **d**, GST interaction assays were performed as described above using GST fusion proteins of wild-type and mutant full-length T₃Rβ1 and the C terminus of T₃Rβ1 (amino acids 186–456) and ³⁵S-labelled *in vitro* transcribed/translated N-CoR C terminus. The lane showing 10% input is indicated. Only the mutant AHT (at 223,224,227)→GGA (T₃Rβ1-AHT_M) of T₃Rβ1 was not able to interact with the C terminus of p270. **METHODS.** **b**, Oligonucleotides used for site-directed mutagenesis were: mutant TEE 210,213,215→RGG: 5'-CACAAGCCAGAGCCAGACGGG-GAATGGGGGCTCATCAAACTGTC-3'; mutant E215→R: 5'-ACAGACGA-GGAATGGCGGCTCATCAAACTGTCAC-3'; mutant EL215,216→GG: 5'-ACAGACGAGGAATGGGGGCTCATCAAACTGTCACC-3'; mutant EL216,221→PP: 5'-GACGAGGAATGGGAGCCCATCAAACTGTCAACCCCA-GCCCATGTGGCGAC-3'; mutant AHT223,224,227→GGA: 5'-AAAACCT-GTCACGAAGCGGTGTGGCGGCAACGCCAAGGC-3' (mutated bases are underlined). The mutations were generated in the vector pBKS. **c**, Gel shift assays were performed with wild-type and mutant T₃Rβ1 and RXRα proteins generated by *in vitro* transcription/translation. The ³²P-labelled DR + 4 site was generated by annealing of two oligonucleotides (sense: 5'-GGGAGCTCGGATCCAGGTACAGGAGGTCAAGATCTGTGCA-CGG-3'), labelled with ³²P-ATP, and incubated with the *in vitro* translated T₃Rβ1 and RXRα proteins. **d**, Using PCR techniques cDNAs were generated encoding GST fusion proteins of full-length WT, mutant E215→R, and T₃Rβ1-AHT_M. A construct coding for a GST fusion protein with T₃Rβ1 (amino acids 186–456) was also generated (see also Fig. 3). GST assays were performed as described for Fig. 3a.

indicate that the TFIIIB interaction cannot alone be sufficient to mediate repression, consistent with the thyroid-hormone-receptor C-terminal transfer experiments of Baniahmad *et al.*²¹. Rather, ligand-independent repression by retinoic-acid and thyroid-hormone receptors requires the recruitment of the co-repressor N-CoR (Fig. 6c), which itself harbours a transferable repressor domain. This recruitment-based repression is reminiscent of the mechanisms of repression by HLH heterodimers in interactions with 'groucho' in *Drosophila*⁴⁰ and by MATα2/MCM1 via recruitment of the SSN6/TUP1 repressor in yeast^{41,42}, and by E2F via recruitment of the retinoblastoma protein⁴³. Indeed, N-CoR and other co-repressors may well prove to use similar molecular mechanisms to achieve their repressor functions with diverse arrays of transcription factors.

N-CoR specifically interacts with a region in the hinge between the DNA and the ligand-binding domain that is well conserved between all thyroid-hormone and retinoic-acid receptors, identifying a novel regulatory domain in what was previously considered to be a poorly conserved region in the C terminus of nuclear receptors. Indeed, by Chou-Fasman and Garnier-Osguthorpe-Robson algorithms, this region is suggested to form an amphipathic helix, and it is likely that coiled-coil interactions with a potentially helical region within the 45-amino-acid interaction domain of N-CoR are required for the high-affinity protein-protein interactions within the cell. Because N-CoR fails to interact with the vitamin D receptor, which is highly conserved within the CoR box, there are probably additional requirements for high-affinity interactions within the N-terminal portion of the ligand-binding domain. The observation that ligand does not cause dissociation of N-CoR from the thyroid-hormone and retinoic-acid receptors in solution indicates that the hinge and ligand-binding domains must undergo specific conformational

changes upon binding of heterodimers to DNA which permit ligand to effect the release of N-CoR. Indeed, Kurokawa *et al.*³² show that this specific conformational change is dependent on the configuration of the DNA-binding site to which RAR/RXR heterodimers bind. On DNA sites on which RAR exerts a constitutive repressor function, such as the retinoic-acid-response element in the *CRBP1* gene⁴⁴, ligand fails to cause the dissociation of N-CoR.

The finding that N-CoR interacts with the thyroid-hormone and retinoic-acid receptors through a region that is distinct from that required for co-activator interaction raises a number of questions. Because the dissociation of N-CoR in response to ligand only occurs when the receptors are bound to positive DNA-response elements, it is possible that when it is no longer bound to DNA, ligand could promote the simultaneous association of N-CoR/receptor complexes with co-activators. Such interactions might serve to recruit N-CoR to promoters and provide an explanation for ligand-dependent repression of specific target genes. Because the retinoid-X receptor can form heterodimeric interactions with other nuclear receptors that do not interact with N-CoR but also bind with high affinity to the same DNA sequences as RXR/RAR or RXR/T₃R heterodimers, it is likely that the balance between positive and negative regulation of transcription is determined not only by the availability of ligand, but also by heterodimer composition. For example, the orphan nuclear receptor, LXR (ref. 45), which forms heterodimers with RXR on a subset of T₃ response elements, does not mediate repression and is permissive to RXR-dependent transactivation. Conversely, T₃Rα2, an alternatively spliced product of the thyroid-hormone-receptor-α gene⁴⁶ which contains all the information necessary for interaction with N-CoR but lacks sequences required for co-activator interaction, functions as a

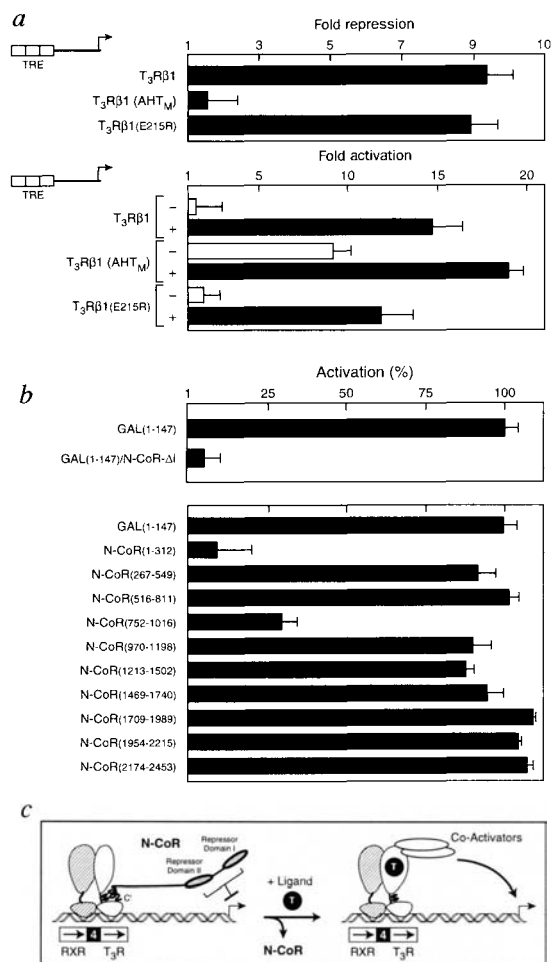


FIG. 6 N-CoR is a co-repressor required for ligand-independent repression function of the T₃ receptor. **a**, Luciferase reporter genes under the control of the minimal tk promoter (−37 shown), or a longer tk promoter (−157 n), with three copies of the myosin heavy chain T₃ response element, were used to assess the repression function of wild-type or mutant T₃ receptors. T₃β1 receptors harbouring the N-CoR box mutant AHT_M lose ligand-independent repression function. The ability of the mutant receptors to activate transcription was assessed on addition of ligand, as shown in the lower panel. Here, the level of T₃-dependent stimulation reaches hybrid levels with T₃Rβ-AHT_M, confirming its ability to act as a ligand-dependent activator. **b**, N-CoR protein can function as a repressor when tethered to DNA and contains a major N-terminal repressor domain. A transcript encoding an N-CoR, GAL4 fusion protein without the C-terminal interaction domain (N-CoR ΔC) was placed under control of the CMV promoter, and assessed for function with promoters controlled by GAL4 binding sites (upper panel). Fragments encoding overlapping regions of 300–350 amino acids, encompassing the entire N-CoR sequences, were fused to GAL4 DNA-binding domains to assess their potential to transfer activation or repression. The activity of each construct was compared to the GAL4(1–147) (−150,000 LU). A major repression domain was identified in the N-terminal 300 amino acids, and a second transferable repressor domain (residues 752–1,016) was also detected. **c**, Model of co-repressor modulation of thyroid hormone receptor repressor function. We suggest that a 270k Co-repressor interacts with the T₃ hormone receptor hinge domain, in a region referred to as the CoR box, in the RXR/T₃R heterodimer bound to cognate T₃ DNA response elements. This interaction involves a 45-amino-acid C-terminal domain on N-CoR, probably via a coiled-coil interaction as indicated. The N-terminal transferable repression domains of N-CoR are postulated to modulate repression function. On binding of ligand, N-CoR dissociates from DNA-bound thyroid hormone receptor, and binding of co-activators permits activation function.

METHODS. **a**, **b**, Co-transfection experiments were conducted using a transcription unit expressing a N-CoR-GAL4 fusion (GAL4 replacing the interaction domain) and various PCR-generated N-CoR fragments fused to the GAL4 DNA binding domain. Expression was 56,000 light units in the absence of T₃R. The luciferase reporter plasmid used in the transfections contained a single GAL4 binding site (17-mer) inserted at position −37 of the thymidine kinase promoter. Cells were transfected with 1 μg GAL-37tk-Luc reporter construct, 0.5 μg expression vector directing the synthesis of the various GAL-N-CoR fusion proteins, and 1.5 μg CMV-LacZ as an internal control. Luciferase activity of the promoter construct with pCMX-GAL4(1–147) alone was calculated as 100% (∼156,000 LU).

constitutive repressor⁴⁷. This compares with the opposite transcriptional activities of Myc/Max and Mad/Max heterodimers. Exchange of Mad for Myc switches Max heterodimers from transcriptional activators to repressors owing to the recruitment by Mad of the co-repressors mSin3A and mSin3B^{48,49}.

Because the repressor function of N-CoR appears to be dominant to ligand-dependent transcriptional activation events, and N-CoR is not displaced in the presence of ligand on certain

DNA sites, the regulation of N-CoR itself will serve to control the expression of nuclear receptor target genes. These data imply that N-CoR may be critical for developmental and homeostatic functions. An accompanying manuscript⁵⁰ describes a related co-repressor with a conserved nuclear receptor interaction domain (SMRT). We suggest that the two related factors be termed TRACs, for thyroid-hormone- and retinoic-acid-receptor-associated co-repressors. □

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LETTERS TO NATURE

Constraints on the origin of the Moon's atmosphere from observations during a lunar eclipse

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THE properties of the Moon's rarefied atmosphere, which can be traced through observations of sodium and potassium^{1–4}, provide important insights into the formation and maintenance of atmospheres on other primitive Solar System bodies^{5–7}. The lunar atmosphere is believed to be composed of atoms from the surface rocks and soil, which might have been sputtered by micrometeorites⁸, by ions in the solar wind⁹, or by photons^{10,11}. It might also form by the evaporation of atoms from the hot, illuminated surface^{10,11}. Here we report the detection of sodium emission from the Moon's atmosphere during a total lunar eclipse (which occurs when the Moon is full). The sodium atmosphere is considerably more extended at full Moon than expected—it extends to at least nine lunar radii—and its brightness distribution is incompatible with sources involving either solar-wind or micrometeorite sputtering. This leaves photon sputtering or thermal desorption as the preferred explanations for the lunar atmosphere, and suggests that sunlight might also be responsible for the transient atmospheres of other primitive bodies (such as Mercury).

To test hypotheses about the source of the lunar atmosphere, observations under different lunar phase conditions are required to help sort out geometrical factors that link sources and resultant morphologies. For example, the Earth's magnetosphere shields the lunar regolith from direct solar-wind impact for the three to four days spanning full Moon each month. Potter and Morgan⁹ reported spectroscopic observations that suggested a dramatic decrease in the sodium brightness at low altitudes (<100 km above the surface) during periods near full Moon, thereby implying that the solar-wind-sputtering source is very important.

Whereas high-resolution spectroscopy and very narrow field (3–4 arcmin) imaging¹² can monitor the sodium distribution close to the Moon under essentially all lunar phase conditions, this is not the case for wide-angle (6–7°) two-dimensional-imaging investigations of the extended atmosphere. With our technique of keeping the disk of the Moon behind an occulting mask and using ~14-Å-wide interference filters, successful images have been obtained only on days near quarter Moon¹³. For the crescent Moon (new±three days), bright sky conditions due to the Moon's proximity to the Sun and long path lengths through the terrestrial atmosphere have, to date, prevented acquisition of images. Under full-Moon conditions, scattered light from the terrestrial atmosphere is simply too strong and attempts to obtain wide-field images have not been successful.

Faced with the severe observational restrictions imposed by scattered light, we recently examined the possibility of acquiring

images of the lunar atmosphere under full-Moon conditions when the lunar surface is in the Earth's shadow, but the sodium atmosphere is still in sunlight. This is the situation during the total phase of a lunar eclipse; scattered moonlight ceases to be a problem (the brightness of the eclipsed Moon is at least 10⁴ times fainter than the full Moon), and thus the extended lunar atmosphere becomes visible in much the same way as a solar eclipse reveals the corona. An opportunity to test this approach occurred on 29 November 1993 when a total lunar eclipse occurred near zenith at the McDonald Observatory in Fort Davis, Texas.

Figure 1 shows the geometry of the eclipse as viewed from that site. During the total phase, a sequence of in-band (Na D₁ + D₂) images at 5,893±7 Å and out-of-band (6,200 Å) images were taken. Exposure times using a bare CCD detector were 10 min 13 s, commencing at 06:02:33 UT and 06:16:15 UT, respectively. As described in our previous work^{14,15}, the in-band image includes sodium from terrestrial and lunar sources, plus scattered light, whereas the out-of-band image shows scattered light. A subtraction yields sodium-only results, but from both lunar and terrestrial regions. A similar image subtraction, taken with the telescope not pointing at the Moon, shows the terrestrial sodium, and hence a final subtraction yields the circumlunar sodium distribution. In addition to standard dark-noise and flat-field corrections, and unique to an eclipse observation, a correction for the penumbral light curve was applied, as sodium atoms are made visible by resonantly scattered sunlight that decreases in intensity from the outer to inner edges of the penumbral region. The final processed image, photometrically calibrated in units of Rayleighs (R), is shown in Fig. 2.

There are several important features in Fig. 2. First, the size of the lunar sodium atmosphere is clearly detectable out to ~9 lunar radii (R_L), a factor of two larger than obtained for the coma under quarter-Moon conditions¹⁵. At these large distances, the coma exhibits radial symmetry, as shown in fixed radial scan plots below the image. There is a slight asymmetry seen at closer radial distances, but small uncertainties in a host of image-processing corrections (for example, a gradient in the brightness of the terrestrial sodium layer) cause us (at present) to attribute no scientific importance to departures from azimuthal symmetry.

At two positions in the radial scan plots, radial power-law fits to the brightness patterns are given. Under full-Moon conditions, the solar zenith angle $\chi = 90^\circ$ at all locations along the limb, a condition existing only at the poles under quarter-Moon phase. For the eclipse case, the derived equivalent brightness for a light ray just grazing the lunar limb is ~1 kR, with a radial power-law index of ~2. These are remarkably close to the average results at $\chi = 90^\circ$ for the quarter-Moon case¹⁵. Thus although the full-Moon peak brightness levels are considerably lower than those at the sub-solar point at quarter Moon (by a factor of 7), it appears that this is primarily a solar zenith angle and viewing geometry result, and not one caused by the terrestrial magnetosphere's shielding of solar-wind-induced sputtering sources.

The result presented here is in marked contrast to recent findings, including unpublished observations during this eclipse (A. Potter, personal communication), that point to a very significant

decrease in sodium brightness levels under full-Moon conditions. It is tempting, at first, to seek a reconciliation of these divergent views by considering the altitudes sampled. The Potter and Morgan results⁹ come from altitudes very close to the limb (average height, 40 km), whereas the imaging data shown here start at a height of $0.5R_L$ (~ 800 km) and extend to $\sim 9R_L$ ($\sim 14,000$ km above the surface). For a solar-wind source turned off by the Moon's entry into the magnetosphere, Na atoms near the surface might show an immediate decrease (and remain low for three to four days), while Na atoms aloft at great distances would persist. Yet Na is destroyed by photoionization, having a lifetime of only ~ 14 h at 1 AU , and thus it is difficult to attribute the large, bright coma in Fig. 2 to a source that has been 'off' for approximately two days. We conclude that the solar-wind-sputtering source cannot be the dominant agent for the production of the extended lunar atmosphere. As discussed

below, the near-Moon sodium may be dominated by a population of cool atoms whose trajectories would all be behind the occulting mask in our observations. Such a component might well decrease appreciably near the solar terminator, leaving the more energetic, sputtered, atoms to reach to the greater distances sampled by our system¹¹.

We now consider the implications of our observations for the understanding of transient atmospheres in the Solar System. As suggested above, solar-wind sputtering cannot be the dominant agent for the production of the extended lunar atmosphere, simply because the lunar Na coma persists when the Moon is shielded from solar plasma during its passage through the Earth's magnetosphere. The implications for Mercury are straightforward: Mercury has its own magnetosphere to deflect the solar wind, and thus micrometeors, or photosputtering and thermal desorption, remain as possible sources for its atmosphere. Micrometeor sources can be dealt with using the lunar case. Although Monte Carlo simulations show that sources that are spatially uniform^{16,17} or dependent on solar zenith angle¹⁸

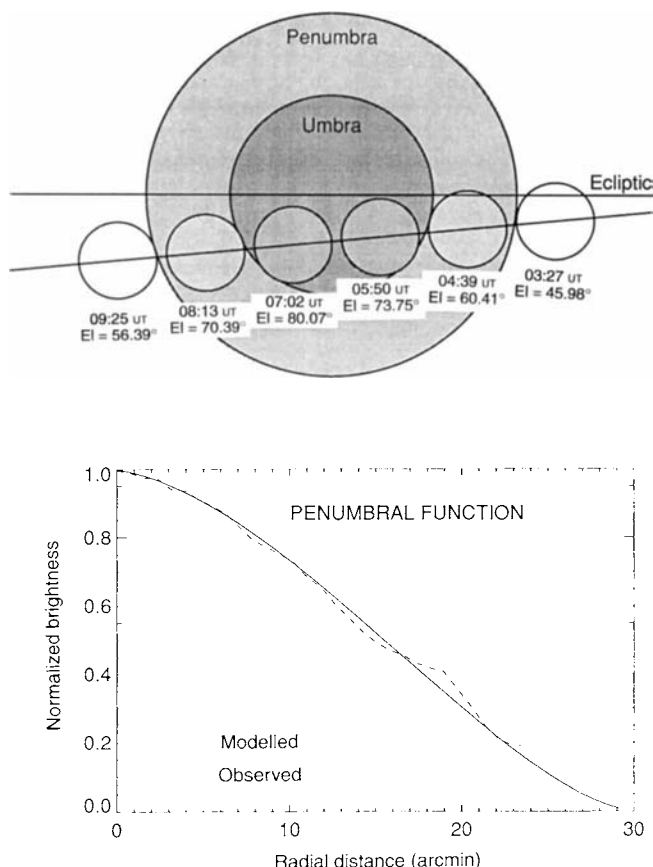


FIG. 1 Top, geometrical and temporal parameters associated with the total lunar eclipse of 29 November 1993, as viewed from the McDonald Observatory (EI is the elevation angle of the centre of the Moon). With almost no sunlight to illuminate sodium atoms within the umbra, there are no usable data from that region. Bottom, in the penumbra, the intensity of sunlight increases to 100% along radial lines from the centre of the umbra (the horizontal axis gives the radial distance from the outer to the inner edge of the penumbra). The observed Na brightness needs to be amplified by this penumbral light function to give the true morphology in the absence of an eclipse. This required function was obtained observationally by dividing a white-light image of the Moon taken when it spanned the penumbra by an image of the fully illuminated disk when the Moon was beyond the umbra, that is, at approximately 08:15 and 09:25 UT. Numerically, this function can also be obtained by having a circular Earth eclipse a solar disk of unit intensity, but with a limb darkening pattern of $1-u[1-\cos \theta]$, where $u=0.6$ and $\theta=0-90^\circ$ from the centre to the edge of the Sun's disk²². Both of these methods gave similar results, as shown, and thus the modelled penumbral function was used to correct those portions of the image affected by the penumbra. Not wanting to amplify any observation by more than a factor of ten, we do not portray results >27 arcmin into the penumbra, where the penumbral light function is <0.1 .

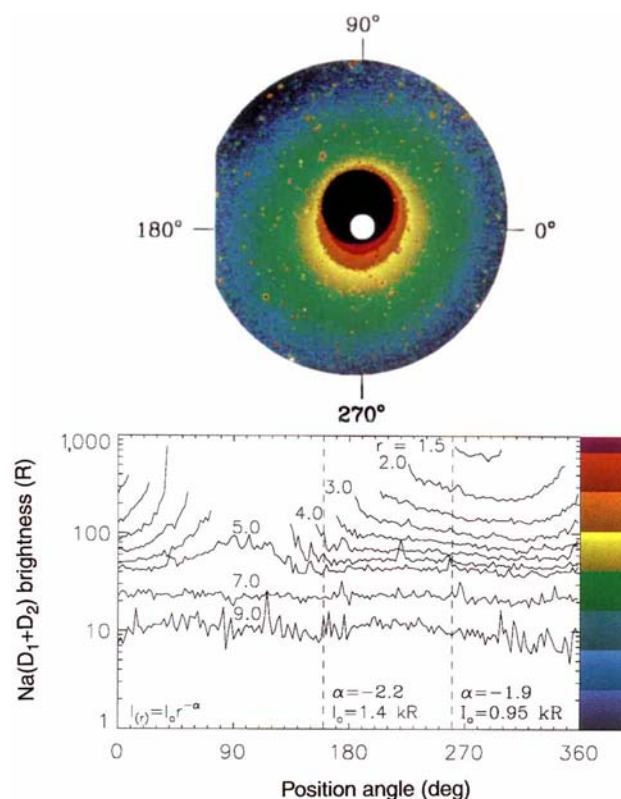


FIG. 2 Top, Sodium brightness surrounding the Moon as obtained during the total phase of the lunar eclipse of 29 November 1993. The dark region in the centre eliminates data from a region where the penumbral function is $\leq 10\%$. The position of the obscured lunar disk is given by the white circle region. The brightness levels are displayed in units of Rayleighs (R), with the colour-coded scale given in the bottom panel. Bottom, brightness scans of the eclipse image at fixed radial distances from 1.5 to 9 lunar radii (R_L) using the position angle system depicted in the top panel. The various offsets of the Moon with respect to the occulting mask and the positions of the umbra and penumbra do not allow for complete azimuthal coverage at all radial distances. Power-law representations of the radial pattern are illustrated at two positions (160° and 260°), showing that the basically symmetrical brightness pattern can be described as $I(r) = I_0 r^{-\alpha} \approx 1kR/r^2$ (where r is given in units of lunar radii, and I_0 is the equivalent brightness at the limb).

can account for the macroscopic effects of a large coma and tail, the uniform-source calculations do not reproduce the observed patterns at quarter Moon¹⁸. Thus, micrometeoroids (if assumed to be a uniform source^{8,16,17}) can be eliminated. An influx that varies with latitude and/or local time, as observed on Earth¹⁹, remains to be examined.

The photosputtering and thermal-desorption mechanisms are driven by sunlight. Thermal desorbed atoms are expected to have low speeds corresponding to typical surface temperatures (~350 K), whereas sputtered atoms would have higher, supra-thermal (~2,000 K) speeds. Thermal and supra-thermal sources would result in atmospheres close to the surface and extended from it, respectively. Such sources act only on the Sun-facing side of the Moon, and they have a solar-zenith-angle dependence: they are greatest at the subsolar point and decrease towards the limb. Compelling arguments have been given that they both act in a 'competing release mechanisms' model^{10,11}. Surface temperatures decrease away from the subsolar point, leading to low thermal source efficiencies near the limb (at the poles for quarter Moon, and all around the disk at full Moon), in agreement with observations close to the surface. The supra-thermal component responsible for the extended atmosphere cannot be uniform as a source peaked at the subsolar point is clearly required to account for the quarter-Moon patterns¹⁸. Although details of a photosputtering source are still not fully known, and more observations are needed to clarify the behaviour of competing hot and cold mechanisms^{11,12}, our wide-field observations find the photosputtering mechanism to be an adequate framework in which to account for the morphologies of the sodium atmosphere at both quarter Moon and full Moon.

If the Moon and Mercury indeed have similar surface materials, then photosputtering applied to the planet's dayside, where solar-wind particles cannot hit directly, would produce a source that increases as the inverse square of distance from the Sun (that is, from 1 AU for the Moon to ~0.4 AU for Mercury), resulting in a source at Mercury ~7 times greater than at the Moon. Moreover, the source at Mercury itself would vary as r^{-2} (where r is Mercury's distance from the Sun) due to the planet's elliptical orbit. Support for both effects is given in separate Monte Carlo simulation studies recently published^{17,20}. We suggest here that a long-sought-after synthesis of observations and models linking the two bodies²¹ indeed points to surface erosion mechanisms initiated by sunlight as the unifying approach to understanding their transient atmospheres. □

A geochemical model for the formation of hydrothermal carbonates on Mars

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It is often argued¹⁻³ that substantially more carbon dioxide and water were degassed from the martian interior than can be found at present in the atmosphere, polar caps and regolith. Calculations have shown that atmospheric escape cannot account for all of the missing volatiles⁴. Suggestions that carbon dioxide is stored as marine or lacustrine deposits⁵, are challenged by Earth-based and spacecraft remote-sensing data^{6,7}. Moreover, recent modelling of the martian atmosphere suggests that rainfall or open bodies of water are in any case unlikely to have persisted for extended periods of time^{8,9}. Hydrothermal carbonates therefore provide a possible solution to this dilemma. Using an accessible terrestrial system (Iceland) as a guide to the underlying processes, and a host rock composition inferred from the least-altered martian meteorite¹⁰, we present a geochemical model for the formation of carbonates in possible martian hydrothermal systems. Our results suggest that an extensive reservoir of carbonate minerals—equivalent to an atmospheric pressure of carbon dioxide of at least one bar—could have been sequestered beneath the surface by widespread hydrothermal activity in the martian past.

Hydrothermal systems are an inevitable consequence of igneous activity occurring in the presence of liquid water, and they have presumably existed on Mars, powered by heat from volcanic intrusions and possibly by impact events¹¹. Direct analysis of martian hydrothermal systems is not possible at present, but the study of active, accessible and diverse terrestrial systems, such as those in Iceland, can considerably expand our understanding of martian systems and influence exploration plans.

In Iceland, hydrothermal circulation systems are deep and widespread. The source of water for systems in the interior of the island is meteoric. This relatively fresh water interacts with basaltic host rock at temperatures to at least 340 °C (refs 12, 13). These systems are well characterized chemically, mineralogically and physically, as summarized in Fig. 1, which shows the generalized petrogenetic sequence of the observed alteration minerals found in basalt-hosted systems throughout Iceland as a function of temperature^{12, 17}. Calcite is present over the entire temperature range of alteration. Hydrothermal carbonate deposits in Iceland range from ~1 to ~30 vol.% of the altered rock, with an average of approximately 4% (ref. 18). Carbonates are found as vein-filling materials and mineral replacements, and are located at least as deep as the deepest well (~2 km below the present surface)^{14, 15}. The distribution and ubiquity of the deposits imply that a great deal of carbon dioxide is concealed below the surface as hydrothermal deposits. Assuming 50% alteration to a depth of 5 km and 4 vol.% carbonate in the alteration, we estimate that a likely inventory of CO₂ sequestered within Iceland is ~2.5 mbar or about 6 times the 0.4 mbar of CO₂ in Earth's present atmosphere. This entire amount of CO₂ would have been sequestered over only the past 16 Myr, Iceland's lifetime¹⁴. This calculation demonstrates that hydrothermal systems, even on a small scale, can greatly affect a planet's CO₂ inventory.

To consider the generation of Icelandic deposits in more detail, we have pursued a series of reaction-path calculations¹⁹ involving host rock composition, temperature, water:rock (W/R) ratio, and carbon dioxide fugacity as variables. These calculations start with a system far from equilibrium and use

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irreversible thermodynamics to evaluate the geochemical consequences of progress toward an equilibrium state. In the course of the calculations, the effects of fluid interaction with the host rock and subsequent alteration phases are monitored in terms of reaction progress. These theoretical results are independent of spatial and temporal constraints, but can be 'mapped on to' geological systems. In these calculations, conducted with the program EQ3/6 (ref. 20), we used three different Icelandic host rock materials (tholeiite basalt, andesite and rhyolite²¹) at three temperatures (50, 150 and 250 °C), and three time-integrated W/R ratios (1, 10 and 50 by mass). Results consistent with the deposition of carbonates are found at all temperatures, rock types and W/R ratios; but as temperature increases, the fugacity of carbon dioxide (f_{CO_2}) must be higher to stabilize carbonate minerals.

Results presented here for Iceland are those most easily compared with results from Mars (basaltic host rock, meteoric source water and low W/R ratio). As an example, Fig. 2a, b summarize Icelandic results for basalt alteration at 250 °C with two different CO_2 fugacities. In these calculations, the system is closed for all components except CO_2 , which is maintained at a constant fugacity. Models at a constant f_{CO_2} equivalent to the present terrestrial atmospheric CO_2 partial pressure (Fig. 2a) are consistent with the formation of prehnite [$\text{Ca}_2\text{Al}_2\text{Si}_5\text{O}_{10}(\text{OH})_2$] rather than carbonate [$(\text{Ca}, \text{Mg}, \text{Fe})\text{CO}_3$]. With a moderately higher value of f_{CO_2} , the calculated assemblage includes carbonate (97% CaCO_3), not prehnite (Fig. 2b). This change is consonant with observations from Iceland, where calcite and prehnite assemblages occur in alternating layers, but not commonly together¹³. We believe that cycling of CO_2 through the Icelandic systems causes this layering effect. During periods of volcanic and/or tectonic activity, released magmatic gases become entrained in the hydrothermal fluids, increasing the f_{CO_2} and stabilizing calcite, even at higher temperatures (to at least 340 °C). During periods of quiescence, the f_{CO_2} decreases, and calcite does not form, allowing other calcium-rich minerals such as prehnite to precipitate.

Martian hydrothermal systems could be extremely similar to those Icelandic systems where the source of water is meteoric rather than sea water. To test this hypothesis we chose a host

rock of shergottite composition (a basaltic SNC meteorite¹⁰) for reaction-path calculations using water containing variable amounts of CO_2 (Fig. 2c). The shergottite host was chosen because it appears to be the least altered of the basaltic SNC meteorites and therefore provides a first estimate for a starting composition. Comparison of Fig. 2b, c shows that chlorite, carbonate (96% CaCO_3), tremolite, albite and quartz are common to both the Icelandic and martian calculated alteration assemblages. The differences between the Icelandic and martian results seen in Fig. 2 are generated by differences in initial bulk composition and mineralogy.

Similar calculations demonstrate that the presence of carbonate is plausible under a wide range of likely martian hydrothermal temperatures and CO_2 fugacities, as illustrated in Fig. 3, which can be used to estimate the potential production of carbonate minerals (in mol%) for any combination of temperatures

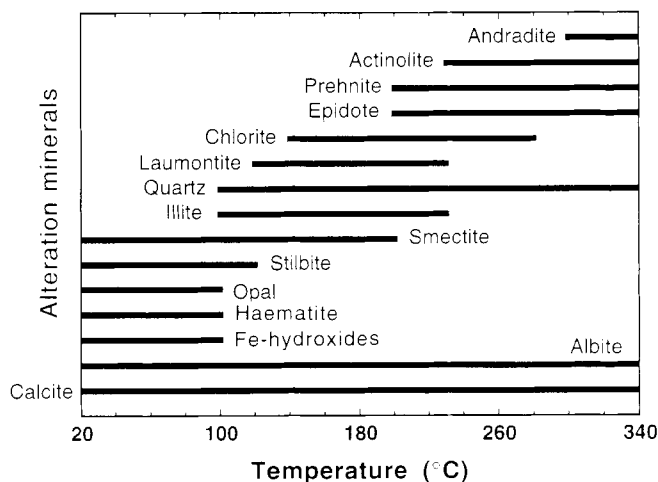


FIG. 1 Generalization of observed Icelandic fresh-water hydrothermal alteration assemblages in basalt as a function of temperature. This figure is a compilation from a number of sources¹²⁻¹⁷. Assemblages can vary slightly in individual locations by the addition of minor and trace phases, for example analcime, anhydrite and pyrite.

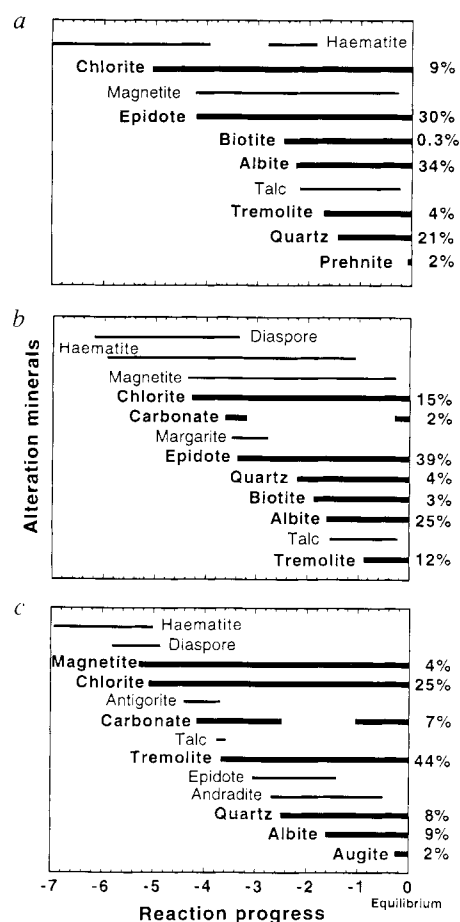


FIG. 2 Predicted alteration assemblages for progressive alteration of Icelandic and martian basalts with water at 250 °C, pressure equal to vapour-liquid equilibrium for H_2O and a W/R ratio of 1 by mass. a, Icelandic results for $f_{\text{CO}_2} = 0.4$ mbar; (atmospheric); b, Icelandic results for $f_{\text{CO}_2} = 2$ bar c, martian results for $f_{\text{CO}_2} = 1$ bar. Reaction-path calculations monitor reaction progress, which is a quantitative measure of the extent to which equilibrium has been reached (see ref. 19). These plots show logarithmic values of the reaction progress variable, which is calculated from the mass of rock that has been altered. A value of -7 pertains to a system of unaltered rock, water and the indicated f_{CO_2} , and a value of 0 denotes the equilibrium state as a result of hydrothermal alteration. Numbers at the right give mass percentages of the minerals in the equilibrium assemblages. The following phases are solid solutions with compositions that may change during the course of reaction progress: albite, augite, biotite, carbonate, chlorite, epidote and tremolite.

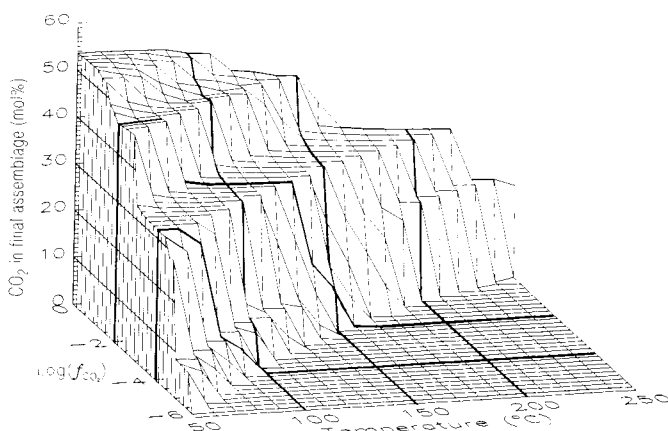


FIG. 3 Carbonate production capacity from a shergottite composition ($W/R=1$) as a function of temperature and constant f_{CO_2} (in bar). The surface represents mol percentages of CO_2 incorporated into equilibrium alteration assemblages as carbonate minerals: calcite solid solution containing variable minor amounts of magnesium and iron and/or dolomite. At higher temperatures a higher f_{CO_2} is required to reach the value of dissolved CO_2 necessary to stabilize carbonate, consistent with the decreasing solubility of carbonate minerals with increasing temperature. The step-like appearance of this surface results from the interplay between the stabilities of the calcite solid solution and dolomite, which are in turn influenced by reactions involving other calcium-, magnesium- and iron-bearing minerals. The third step from the bottom is due to the appearance of dolomite.

(from 50 to 250 °C) and f_{CO_2} (from 10^{-6} to 1 bar). As an example, it can be seen that carbonate can precipitate at temperatures ≤ 150 °C at f_{CO_2} equal to that in the current martian atmosphere ($10^{-2.2}$ bar = 6 mbar). Amounts of carbonate predicted at 150 °C and $\log f_{CO_2} = -2.2$ are small (~ 1 mol%), but would increase considerably at equilibrium if the temperature of alteration were lower for the same f_{CO_2} . If the temperature of alteration exceeds 150 °C, then a higher f_{CO_2} is required for carbonate precipitation, as shown in Fig. 3. As in Iceland, locally higher f_{CO_2} values on Mars may be attained through magmatic outgassing and CO_2 entrainment.

There are several applications of mass-transfer calculations to the study of the evolution of Mars. For example, combining model calculations with petrologic and isotopic studies of martian rocks, including the SNC meteorites, may lead to a better understanding of the sequence of events leading to the present appearance of these rocks. But it is not our aim in this work to

explain the petrogenesis of the SNC meteorites. In the future, combination of more comprehensive theoretical calculations with analytical studies may help to constrain alteration temperatures, scenarios and carbonate production in the SNC meteorites. Our present calculations produce anywhere from minute to massive amounts of carbonate (Fig. 3), but smaller amounts seem more consistent with the few samples of the martian surface currently accessible. Although the calculated assemblages are not wholly reflected in these few samples, this is not an argument against the plausibility of hydrothermal systems having strongly influenced martian volatile processing. Because it is possible that most hydrothermal activity occurred early in Mars' history, samples from that era would be revealing. Unfortunately, only one old sample (ALH84001: > 3 Gyr (ref. 22)) is currently recognized. This particular sample has had an extremely complex history and has left Mars researchers with the problem of trying to piece together the planet's early history from one very confusing example²²⁻²⁵. The carbonate in this specimen is very rich in magnesium and iron, and may reveal much about the composition of martian fluids once the history of the meteorite is better understood.

Finally, these theoretical calculations allow first-order estimation of the amount of CO_2 that could be hidden as hydrothermal carbonate in Mars. We estimate that a maximum of 5 bar of CO_2 could be sequestered in the martian crust owing to hydrothermal alteration. This requires $\sim 2.5\%$ carbonate globally dispersed throughout the upper 5 km of crust. Crustal storage of 1 bar of CO_2 would require an average of only 0.5% carbonate in the upper 5 km or 1.1% in the upper 2 km. Thus we submit that hydrothermal deposits could account for a considerable amount of the carbon dioxide released by volcanic activity on Mars, with many implications for understanding the inventory of martian volatiles. Because magmatic activity was more frequent on Mars in the first half of geological time, we surmise that the effectiveness of hydrothermal systems for sequestering CO_2 has changed dramatically with time. Hydrothermal processes would have been particularly important if Mars did not have a hydrologic cycle with extensive rainfall and runoff, that is, if surface temperatures and pressures did not reach the triple point of water. In areas where the water table intersected the surface, warm, CO_2 -laden fluids should have degassed to produce locally extensive travertine units. These sites provide a test of the hydrothermal carbonate hypothesis. Because most of the activity in hydrothermal systems occurs at depth, investigations of the sub-surface at eroded channels, chasma walls, and craters furnish additional areas of observation and testing of this hypothesis. Hydrothermal systems on another planet offer new probabilities for the synthesis of organic compounds and the possible emergence of life^{26,27}. □

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Zeeman splitting of surface-scattered neutrons

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If a beam of slow neutrons impinges on a solid at grazing incidence, the neutrons reflected can be used to probe the composition and magnetization of the solid near its surface^{1,2}. In this process, the incident and reflected neutrons generally have identical kinetic energies. Here we report the results of an experiment in which subtle inelastic scattering processes are revealed as relatively large deviations in scattering angle. The neutrons are scattered from a ferromagnetic surface in the presence of a strong ambient magnetic field, and exhibit a small but significant variation in kinetic energy as a function of the reflection angle. This effect is attributable to the Zeeman splitting of the energies of the neutron spin states due to the ambient magnetic field: some neutrons flip their spins upon reflection from the magnetized surface, thereby exchanging kinetic energy for magnetic potential energy. The subtle effects of Zeeman splitting are amplified by the extreme sensitivity of grazing-angle neutron scattering, and might also provide a useful spectroscopic tool if significant practical obstacles (such as low interaction cross-sections) can be overcome.

The reflecting sample was a cobalt thin film in an unusual geometry. The Co film, 800 Å thick, had been sputtered on a Si substrate and covered with a 1,000-Å layer of deuterated polystyrene. Magnetic fields of up to 13 kOe were applied perpendicular to the surface; the incoming neutrons were polarized³ either parallel (+) or antiparallel (−) to the field. The object was to measure the chemical and magnetic depth profile with Co magnetized perpendicular to the plane. The minimum field necessary to align Co completely perpendicular to the surface is 18 kOe; as a result, the Co magnetization was never completely out of the film's plane. But the experiment yielded an unexpected result.

The measurements were taken at a reflectometer (called POSY I) installed at the Intense Pulsed Neutron Source at Argonne National Laboratory. A polychromatic neutron beam cooled to 20 K (effective bandwidth 3–14 Å) is incident on the sample at an angle θ_i of the order of 1°. A position-sensitive detector measures the neutrons specularly reflected at $\theta_r = \theta_i$ as well as the neutrons scattered at $\theta_r \neq \theta_i$. The detector (Fig. 1) effectively integrates over ϕ , the sagittal angle of the scattered neutron. At

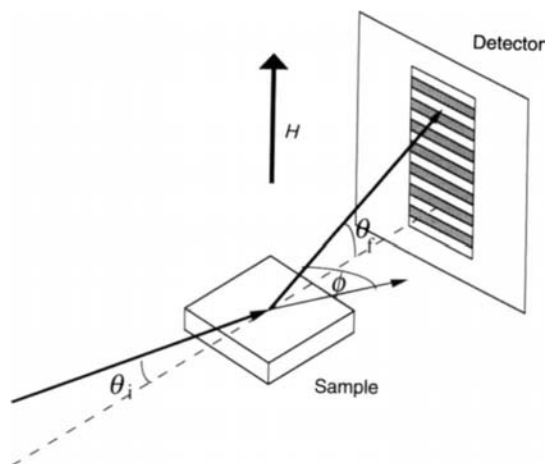


FIG. 1 Geometry of the sample-detector system.

each element of the detector the neutrons are measured as a function of their time of flight from the source, which is proportional to the neutron wavelength³.

We expected to see a ridge of non-zero intensities for all wavelengths λ at an angle $2\theta_i$ to the incoming beam. The normalized intensities constitute the Fresnel reflectivities of the sample, and from them it is possible to reconstruct the chemical and magnetic depth profiles of the sample². Figures 2 and 3 give contour plots of the raw intensities at different magnetic fields and at an angle of incidence $\theta_i = 0.44^\circ$. The ridge at $2\theta_i$ is clearly visible. In addition, a second ridge appears at angles greater than or less than $2\theta_i$ for spin (+) and spin (−) neutrons respectively. Both ridges are dispersive in λ , and the dispersion increases with field.

The strong dependence of the effect on spin and magnetic field led us to look for an interpretation in terms of the Zeeman energy of the neutron spins. The total energy of a neutron in a magnetic field is the sum of its kinetic and magnetic (Zeeman) energies,

$$E = \frac{1}{2}mv^2 \pm \mu_n \cdot H \quad (1)$$

where m is the neutron's mass, v its velocity (related to its wavelength by $v = h/m\lambda$), μ_n the absolute value of the neutron's

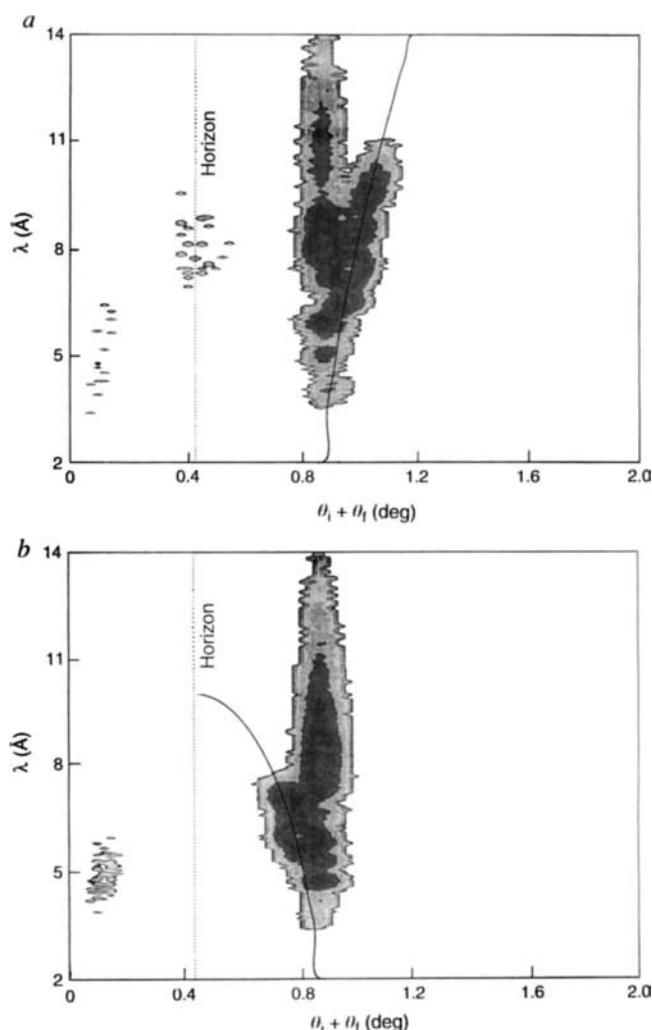


FIG. 2 Contour plots of raw neutron intensities reflected from a cobalt film. The angle of incidence is $\theta_i = 0.44^\circ$, and the neutron wavelengths are in a spectrum ranging from 3 to 14 Å. a, b, Plots for neutron polarized parallel (+, a) and antiparallel (−, b) to a magnetic field of 4.3 kOe applied perpendicular to the surface. The solid lines are calculated from equation (4). The horizon is an ideal line parallel to the sample's surface. In this field, the Zeeman energy, $2\mu_n H$, is 0.05 μeV.

netic moment, and the sign indicates the neutron polarization relative to the magnetic field H . In this, as in the following equations, the upper sign refers to neutron spins (+), the lower to neutrons spin (-).

On striking the sample, the neutrons may see a local magnetic field that is not aligned with the ambient field. The magnetic interaction between the neutron and this local field causes some neutron spins to flip on reflection. The total energy is conserved if

$$k_{zi}^2 + k_{xi}^2 \pm 8\pi^2 m \mu_n H / h^2 = k_{zf}^2 + k_{xf}^2 \mp 8\pi^2 m \mu_n H / h^2 \quad (2)$$

In equation (2) h is the Planck's constant and $k_x = 2\pi \cos \lambda$, $k_z = 2\pi \sin \theta / \lambda$ are the components of the momentum parallel and perpendicular to the surface for neutrons incident at an angle θ , or reflected at an angle θ_f to the surface. An added constraint is the conservation of the neutron momentum in the plane of the surface:

$$k_{xi} = k_{xf} \quad (3)$$

Combining equations (2) and (3) and using the small-angle approximation we obtain

$$\theta_f^2 \approx \theta_i^2 \pm 1.47 \times 10^{-7} H \cdot \lambda^2 \quad (4)$$

where θ_i , θ_f are in radians, H is in kOe and λ in ångströms. The calculated loci of the spin-flipped, reflected neutrons are drawn in Figs 2 and 3 and match well with the maxima of the observed ridges. We should have liked to confirm the spin-flip character

of the neutrons contained in the dispersive branches directly, but in practice this is awkward to measure. Although conceptually less satisfying, spin analysis performed on the central ridge showed it to be composed entirely of non-spin-flipped neutrons.

Spin-flip reflectivity is an adiabatic process: no energy is exchanged between the neutron and the surface, but a very small amount of the energy of the neutron is switched from potential to kinetic. In our experiment it is readily visible as a large change in the reflected angle. This is a direct consequence of the grazing incidence geometry. The small angle implies that $k_{zi}^2 \approx 10^{-4} k_{xi}^2$ and $k_{zf}^2 \approx 10^{-4} k_{xf}^2$ in equation (2), but equation (3) indicates that terms in k_x can be omitted. Alefeld *et al.*⁴ have measured the Zeeman splitting directly for neutrons of $\lambda_0 = 6.28$ Å in a magnetic field of 20 kOe. The energy difference between spin states was 0.25 µeV and could be detected only with the help of a very elaborate apparatus at the Institut Laue-Langevin in Grenoble. In contrast, if we used the same neutron wavelength and magnetic field in a reflection experiment at an angle of incidence of 0.6° we would have obtained exit angles of 0.85° for spin (+) neutrons, whereas spin (-) neutrons would have emerged parallel to the surface.

Obviously the effect on the reflection angle of the Zeeman energy exchange is not limited to our sample, or to a particular configuration of magnetic fields: it suffices to have a spin-flip reflection in an applied magnetic field, regardless of its orientation. The effect may turn out to be useful in neutron scattering. For instance, a device that separates the spin-flipped neutrons from the non-spin-flipped neutrons without recourse to polarization analysis (and without polarizing the incident beam) is conceivable. Looking beyond technical applications, it must be pointed out that in grazing incidence geometry the reflection angle is extremely sensitive to any inelastic process, such as those resulting from exchange of energy between neutron and surface. It is too early to say whether this could open a new chapter in surface spectroscopy. The weak cross-section, or complications arising when not merely energy, but momentum is exchanged between neutron and surface are difficulties that may stunt its development. □

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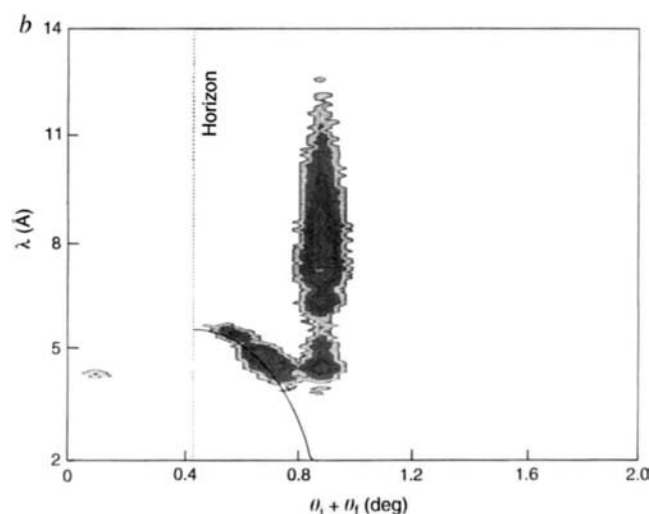
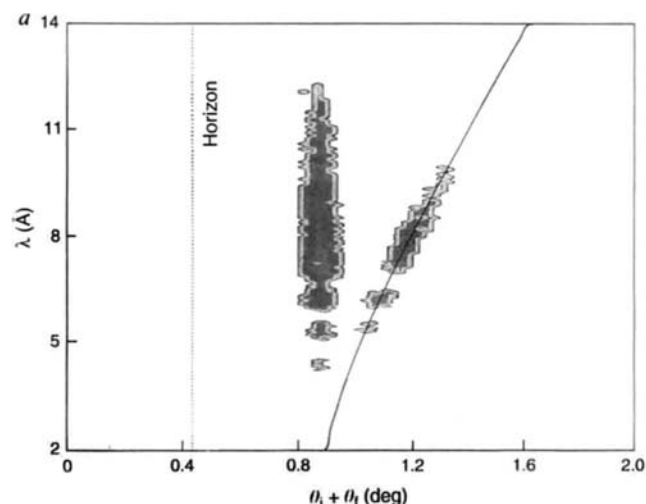


FIG. 3 Similar to Fig. 2, but with a magnetic field perpendicular to the surface raised to 12.7 kOe. Zeeman energy $2\mu_n H = 0.17$ µeV.

Seismic evidence for a magma chamber beneath the slow-spreading Mid-Atlantic Ridge

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SEISMIC reflections from magma chambers have been observed along the fast-spreading East Pacific Rise^{1,2} and the intermediate-spreading Valu Fa Ridge^{3,4}; sub-axial reflections also exist beneath the intermediate-spreading Juan de Fuca Ridge⁵. But no magma chambers have been identified beneath the slow-spreading Mid-Atlantic Ridge, suggesting that here magma chambers lie unusually deep or are transient features⁶⁻¹¹. Seismic reflection profiles acquired in 1989 over the Snake Pit hydrothermal area, in the rift valley of the Mid-Atlantic Ridge ~25 km south of the Kane fracture zone, showed no evidence of magmatic activity¹², although geochemical analyses of hydrothermal vent fluids suggest the existence of magma at depths as shallow as 1-2 km^{13,14}. By suppressing

in these data high-amplitude coherent noise generated at the sea floor, I have obtained images, in an otherwise non-reflective crust, of seismic reflections beneath, and just south of, the Snake Pit hydrothermal area. These reflections define a small, 4-km-wide dome whose apex is $\sim 1,200$ m beneath the sea floor. As bright reflections from the upper flanks of this dome occur in the depth range suggested by the vent-fluid geochemistry, I interpret the dome to be the seismic expression of a small magma chamber.

The axial valley of the MARK (Mid-Atlantic Ridge at Kane) area lies south of the Kane fracture zone and has been the object of a number of geological, geochemical and geophysical surveys. These have included dredging¹⁵, submersible dives¹⁶, fluid sampling¹⁴, side-scan sonar¹⁷, seismic refraction¹⁰, potential field studies^{18,19} and shallow drilling²⁰, making the MARK area the most intensively studied region of the Mid-Atlantic Ridge (MAR). Both geological and geophysical studies support the division of the MARK area into two regions separated by a small non-transform offset near $23^{\circ} 15' \text{ N}$. Analysis of both gravity and seismic refraction data indicate that the crust in the northern zone is $\sim 4\text{--}5$ km thick compared with ~ 6 km further south^{10,19}. The southern area is characterized by a number of sediment-covered, 50–100-m-high volcanic cones within the median valley, suggesting little recent volcanic activity. In contrast, an approximately 40-km-long neovolcanic ridge, which rises up to 600 m above the surrounding valley floor, exists in the northern zone. Exposed, sediment-free pillow basalts imply that the ridge is at most a few thousand years old¹³. A large, active hydrothermal vent field, the Snake Pit hydrothermal area, occurs on the neovolcanic ridge in an area of locally elevated topography ~ 25 km south of the Kane fracture zone²¹. Analyses of fluid samples obtained from vents in the Snake Pit vent field imply that high-temperature geochemical reactions, possibly linked to a crustal magma chamber, occur at depths shallower than 1–2 km below the sea floor¹⁴.

Two orthogonal, multichannel seismic reflection lines were acquired across the Snake Pit hydrothermal field in 1989. Despite the robust nature of magmatic activity in the area, no high-amplitude, laterally continuous sub-axial reflections indicative of a large, steady-state crustal magma chamber were detected¹². Both seismic profiles were shot with an 87.6-litre (5,346 in³) airgun array, and recorded using a 96-channel digital hydrophone streamer with 25 m group interval and 2,700 m far offset. In the absence of any significant sedimentation, the large seismic velocity contrast at the sea floor and the very rough topography combine to produce particularly high-amplitude scattered arrivals in both seismic profiles^{22–24}. Some diffractions possess high stacking velocities and in deep water the difference in normal moveout (NMO) between crustal reflections and sea-floor diffractions is small enough that conventional stacking and the use of frequency–wavenumber velocity filters does not provide adequate attenuation of this coherent noise. As a result, only strong, laterally continuous crustal reflections with reflection coefficients >0.14 can be observed in seismic data processed in this fashion, and smaller magma bodies may remain undetected¹². But the application of a conventional two-dimensional dip moveout (DMO) correction to a straight seismic reflection profile shot over rugged sea floor topography will collapse the stacking velocities of both in-line and out-of-plane sea floor diffractions²⁵ to $1,500 \text{ m s}^{-1}$, creating a significant difference between the stacking velocities of crustal reflections and sea-floor-generated coherent noise that can be exploited by conventional velocity filters²⁶. I have used this approach to reprocess the data from part of line 841 (Fig. 1) along the MAR median valley over the Snake Pit hydrothermal area.

To verify that reflections in the final section do not arise from residual noise remaining in the data after processing, semblance velocity spectra²⁷ (as described in Fig. 2) were computed along line 841. Figure 2 shows the semblance velocity spectrum of DMO corrected common midpoint (CMP) gather 1106 located just south of the Snake Pit area before and after decomposition

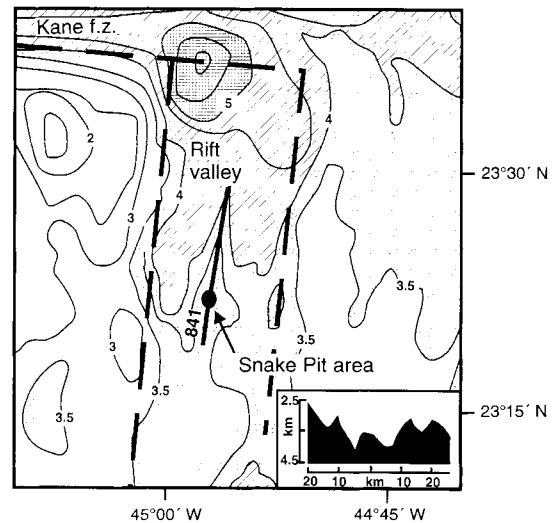


FIG. 1 Sea-floor topography (in kilometres below sea level) of the northern MARK area of the Mid-Atlantic Ridge^{12,17}. The approximate location of the axial valley and its intersection with the Kane fracture zone are indicated by heavy broken lines. The location of the section of seismic profile 841 presented here is shown. The Snake Pit hydrothermal area lies on a region of locally elevated topography on the neovolcanic ridge that occupies the centre of the rift valley and rises up to 600 m above the valley floor. The inset shows an east–west bathymetric profile through the Snake Pit field.

into sea-floor diffractions and underlying primary reflections. The DMO correction has reduced the stacking velocity of sea-floor diffractions, which dominate the section, to $1,500 \text{ m s}^{-1}$ (blue line in Fig. 2a, b). After their removal, some reflections exhibit high stacking velocities consistent with an origin within the crustal section (Fig. 2c). The semblance peaks show the loss of velocity resolution with increasing travel time that is associated with increasing depth to the reflector, and correlate well with the root mean square (r.m.s.) velocity function derived from a seismic refraction survey 5 km to the east¹⁰. Reflection semblance peaks at 6.1 s and 6.7 s are also weakly visible in Fig. 2a, demonstrating that they are not artefacts of the velocity filtering. Stacking velocities of $\sim 3,500 \text{ m s}^{-1}$, consistent with a crustal interval velocity of $5,860 \text{ m s}^{-1}$ derived from the refraction data, were also obtained from two deep reflection packages, one dipping shallowly to the south from 7.0 s at CMP 100 to 7.2 s at CMP 600, the other near CMP 1400 at 6.7 s (M1 and M2 in Fig. 3). The stacking velocity analyses demonstrate that the interpreted reflections, although sometimes weak, do originate within the oceanic crust.

Perhaps the most striking feature of line 841 between CMP 100 and 800 is the absence of strong, coherent energy at mid-crustal depths, in marked contrast with earlier results and implying that most of the interfering coherent noise has been removed (Fig. 3). A packet of weak reflections (M1) is visible, rising slightly to the north between CMP 600 and CMP 100 just below 7.0 s. Using the interval velocity of $5,860 \text{ m s}^{-1}$, the base of M1 is estimated to shoal from 6.7 km to 5.2 km beneath the sea floor towards the north. These depths place the reflections close to the base of the oceanic crust, and they are interpreted as being associated with the crust–mantle transition (Moho) because reflections from the Moho are commonly observed to be the strongest sub-horizontal, sub-basement reflections in oceanic crustal surveys. Moho reflections are probably produced by interlayered mafic and ultramafic rocks within the transition zone, but direct inferences of the location of the transition to mantle seismic velocities for comparison with seismic refraction results can only be approximate²⁸. Nevertheless, the decrease in reflector depths towards the north end of line 841 is consistent

with known thinning of the crust to <2–3 km beneath the intersection of the axial valley with the Kane fracture zone 12 km further north²⁹.

Reflection zone M1 rises sharply at CMP 650 to ~6.2 s beneath the higher topography on which the Snake Pit hydrothermal area is located, implying a reduced crustal thickness of ~4.8 km. A shorter reflection package (M2) is observed at 6.8 s near CMP 1400, indicating that the Moho lies deeper, at a depth of ~5.9 km, south of the hydrothermal field and in agreement with the earlier interpretation¹². Although the crust–mantle transition usually appears to be a relatively smooth reflection in oceanic lithosphere created at faster spreading ridges, there is evidence of significant crustal-scale faulting and Moho topography in the case of slow-spreading ridges^{30,31}. The exposure through faulting of cumulate gabbros and serpentinized peridotites in the northern part of the MARK area^{13,32} suggests that the Moho may well be offset by faults in this region, consistent with the sudden changes in Moho depth imaged in the seismic data. The crustal thickness of 5 km near the Snake Pit area estimated from the Moho reflections is at the high end, although broadly consistent with the range of values estimated from both refraction¹⁰ and gravity¹⁹ data analyses of the northern MARK area.

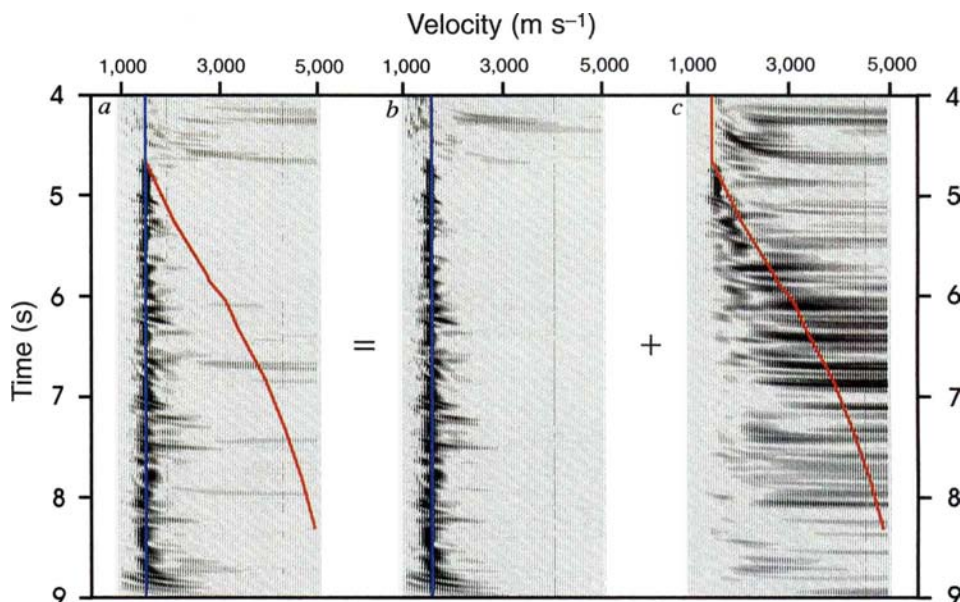
In contrast with the lack of reflectivity in the middle crust between CMP 100 and CMP 800, reflections are observed between CMP 1000 and 1300 (Fig. 3). Stacking-velocity analysis demonstrates that they originate within the crust, because energy scattered from the sea floor exhibits a stacking velocity of $1,500 \text{ m s}^{-1}$ after DMO correction (Fig. 2). The Snake Pit hydrothermal area occurs at CMP 1025 at the northern edge of this zone of reflectivity. Two steeply dipping reflections, shown by red lines in Fig. 3c, define the flanks of a 4-km-wide antiform whose apex lies as shallow as 5.2 s on the stack section, implying a depth of ~1,200 m beneath the sea floor after migration. Near to the sea floor, these reflections exhibit higher amplitudes (thicker red lines), a characteristic of reflections from magma chambers on faster-spreading ridges^{2,4}. Because the two reflections define the upper part of the antiform, a zone of anomalous seismic velocity or density must exist between them, forming a cap. These stronger reflections also occur at depths correspond-

ing to the 'high-temperature reaction zone' inferred from geochemical analyses¹⁴ to exist beneath the hydrothermal field, and interpreted as indicating the presence of melt. Although solidified gabbroic intrusions in the upper crust or hydrothermal alteration along fault zones might create zones of seismic reflectivity, they would neither occur in the form of a dome as imaged in these seismic data, nor produce the shallow, high-temperature geochemical anomaly. A diapir of serpentinized mantle might create the antiform shape, but not vent fluids as hot as 320°C (ref. 14). Thus I interpret the antiform, or dome-like structure, to be the upper surface of a small magma chamber.

Previous geological and geochemical evidence of recent vigorous volcanic activity along the neovolcanic ridge imply the existence of melt at shallow levels within the crust, but do not necessarily imply the presence of a magma chamber. The seismic data suggest the existence of a small lens of molten, or partially molten, material lying between the strong reflections 3 km south of the Snake Pit vent field. This melt zone extends <2 km along the MAR rift valley, on the basis of estimates of the distance between the lower ends of the high-amplitude reflections after migration. Short, sub-horizontal reflections occur between CMP 1,000 and CMP 1300 at 5.5 s, 2,400 m beneath the sea floor, and continue to the inferred base of the crust. These reflections do not extend to the north and may be associated with the magma chamber, perhaps representing layered cumulates. The magma chamber occurs beneath, and close to the centre of, the highest region of the neovolcanic ridge, suggesting that it may have been inflated by the localized melt injection.

Deep, crustal-scale faulting, implied by the widespread recovery of mantle rocks within, and to the west of, the northern MARK area^{13,32}, would allow magma to rise to relatively shallow levels within the crust. In fact, the reflection that forms the southern flank of the antiform extends at least to the lower crust and may represent a fault (F1 in Fig. 3c), possibly associated with the change in Moho depth near CMP 1400; the fault could have played a role in the formation of both the magma chamber and hydrothermal vent field. It is possible that along the slow-spreading MAR the location of high-temperature hydrothermal areas is associated with deep faulting that allows magma to rise

FIG. 2 Semblance velocity spectra calculated at CMP 1106 (V in Fig. 3) from DMO-corrected data before and after separation into sea-floor diffractions and weaker crustal reflections. In a CMP gather, a reflection or diffraction appears as a near-hyperbola, which can be characterized by its zero-offset travel time and a velocity value that indicates the degree of curvature. This 'stacking velocity' approximates the r.m.s. velocity above a reflector. The variation of reflection coherence, or semblance, with zero-offset travel time and stacking velocity can be displayed in a velocity spectrum²⁷ as shown here. The semblance maxima (black peaks) indicate the stacking velocity of a particular reflection. a, Velocity spectrum of DMO-corrected CMP gather showing that the stacking velocities of sea-floor diffractions have been reduced to $1,500 \text{ m s}^{-1}$ (blue line). Two very weak semblance peaks near $3,000 \text{ m s}^{-1}$ are just visible at 6.1 s and 6.7 s, close to the red line that represents the r.m.s. velocity function derived from the nearby refraction velocity model¹⁰. b, Velocity spectrum of sea-floor diffractions after velocity filtering exhibiting stacking velocities of $1,500 \text{ m s}^{-1}$ (blue line). c, Velocity spectrum of reflections possessing high stacking velocities inconsistent with sea-floor-generated coherent noise after DMO correc-



tion. (DMO correction also renders the stacking velocities of crustal reflections independent of dip²⁵.) The refraction model r.m.s. velocity function (red line) matches closely the semblance maxima obtained from the reflection data. This implies that the reflections imaged by reprocessing seismic line 841 originate within the oceanic crustal section.

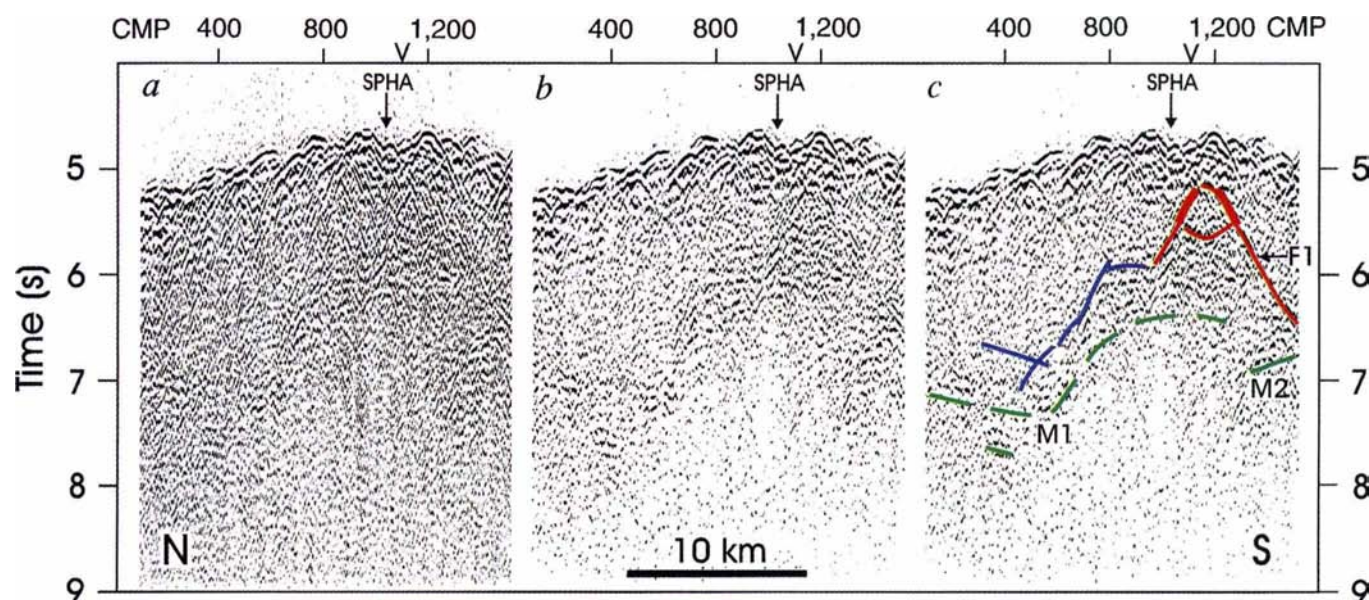


FIG. 3 Stacks of part of seismic profile 841 over the Snake Pit hydrothermal area. Preprocessing of the data included despiking, sorting of the data into 14.5-m CMP bins and amplitude recovery with a gain proportional to time. Subsequently, DMO processing and a velocity filter based on the generalized Radon transform³⁴ were applied. Residual noise on some inside traces was suppressed with an inside-trace mute before stacking. The data are filtered at 3–18 Hz and displayed with no gain at a vertical exaggeration of 2:1 at 6,000 m s⁻¹. a, Stack (without DMO) dominated by coherent noise. Some reflection energy from within the crust is faintly visible. The velocity filter applied to each CMP gather has removed coherent energy exhibiting low stacking velocities that originates mainly out of the plane of the seismic profile. The steeply dipping diffractions that remain possess higher stacking velocities, and have not been suppressed. b, Stack (with DMO). The upper and middle crust appear largely transparent with the exception of the region between CMP 1000 and 1300, where crustal reflections are observed rising to within 0.4 s of the sea floor. A deeper set of reflections inter-

preted as the crust-mantle transition is also visible. Application of DMO processing reduced the high stacking velocities of the coherent noise to 1,500 m s⁻¹, allowing it to be attenuated by the velocity filter. c, Interpretation of b. The dome-like structure beneath, and just south of, the Snake Pit hydrothermal area (SPHA) is outlined in red. Reflections that exhibit unusually high amplitudes relative to other crustal reflections are shown with thicker red lines. Calculations of the migrated positions of the flank reflections (red) were made, to derive the estimates of sub-seafloor depth and extent along the median valley. The location of out-of-plane scattered energy modelled in an earlier analysis¹² is shown in blue, and does not correlate with any strong reflections in the section below 6.6 s. However, weak, steeply dipping residual offline energy may exist in the display between CMP 650 and 750 from 6.6 to 5.9 s. F1: steeply dipping reflection, possibly a fault, that penetrates at least to the lower crust; M1, M2 (green): reflections marking the crust-mantle transition zone; V: location of velocity analysis in Fig. 2

close to the sea floor. This is quite different from the East Pacific Rise (EPR), where there seems to be no correlation between vent location, faulting and the magma chamber reflection, although a connection with a very shallow, 0.1-s sub-seafloor, reflection has been proposed³³. Alternatively, F1 might simply be the boundary of the magma body. In either case, the magma chamber does

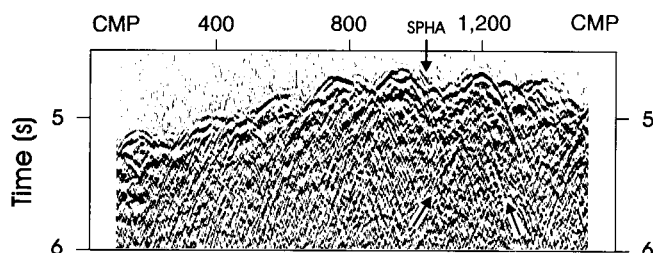


FIG. 4 Stack (without DMO). Processed as in Fig. 3a, but filtered at 3–28 Hz to show better the amplitude variations in the upper crust. The display highlights the stronger reflections from the upper flanks of the magma chamber beneath the Snake Pit hydrothermal area (SPHA). The sea-floor reflection, and (possibly) the reflection from the top of the dome, have been attenuated by the velocity filtering because the stacking velocities of crustal reflections approach those of sea-floor diffractions (1,500 m s⁻¹) at the sea floor. An exact separation of the scattered energy and crustal reflections is impossible immediately below the sea floor.

not extend far along the rift valley and is likely to cool more rapidly than the large chambers found at faster spreading ridges. A cooling magma chamber beneath the Snake Pit hydrothermal area would explain why the reflections observed from its upper boundary are not as strong as those detected at faster-spreading ridges because the contrast in seismic impedance with the surrounding crust could be relatively low. Underlying crustal reflections, perhaps arising from cumulate layering, would thus be more easily imaged. Even the brighter reflections at the apex are significantly weaker than those from the overlying sea floor, in contrast with the EPR where the sea-floor and magma-chamber reflections have comparable reflection coefficients, of ~0.35 (ref. 12).

These seismic results indicate that magma chambers can occur below the slow-spreading MAR at sub-seafloor depths similar to those of faster-spreading mid-ocean ridges. But small magma bodies similar to the one imaged here are likely to be subject to more rapid cooling, implying that they may be transient features. □

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High concentration of atmospheric ^{14}C during the Younger Dryas cold episode

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THE various reservoirs of the global carbon cycle, with their very different residence times, are linked by a complex and evolving system of exchanges for which natural radiocarbon is the most robust tracer¹. Any change in the sizes of these reservoirs, or the exchange rates between them, could perturb the $^{14}\text{C}/^{12}\text{C}$ ratio of each other reservoir, and the smallest of them—the atmosphere—would be the most sensitive. In particular, high-resolution reconstructions of past atmospheric $^{14}\text{C}/^{12}\text{C}$ ratios may provide important clues to the mechanisms of abrupt climate change. Annually

laminated lake sediments potentially provide an optimal record in this respect, as they preserve information about both past atmospheric ^{14}C levels and climate changes, providing absolutely dated material beyond the range of tree-ring chronologies and, unlike corals, directly monitor ^{14}C concentrations in atmospheric CO_2 . Here we report the relationship between atmospheric ^{14}C concentration and climate changes during the Younger Dryas and early Holocene periods, derived from analyses of the annually laminated sediments of Lake Gościąg, in central Poland. We find that atmospheric ^{14}C concentrations during the Younger Dryas were abnormally high, which we interpret as a reduced ventilation rate of the deep ocean, most probably as a result of a decrease in intensity of the North Atlantic Deep Water formation.

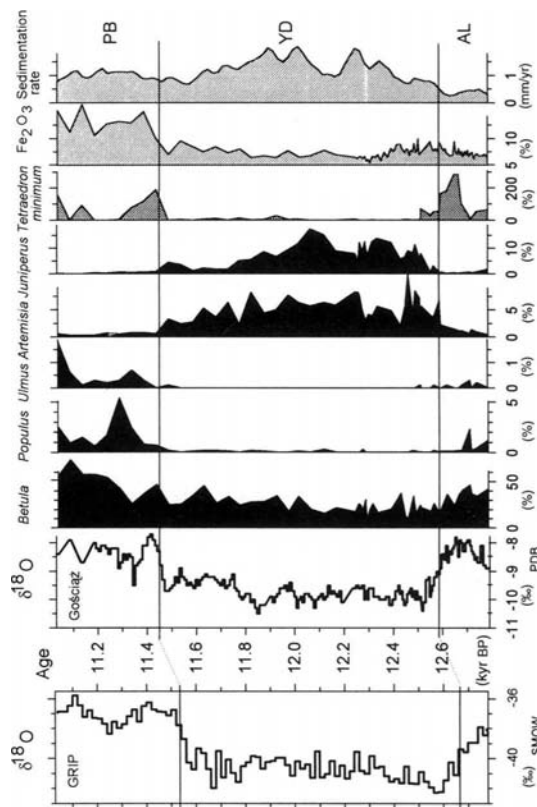


FIG. 1 Combined diagram of proxy climate data recorded in the Late Glacial and Early Holocene part of the Lake Gościąg sediment, compared with the $\delta^{18}\text{O}$ record in the GRIP site, central Greenland. PB, Preboreal; YD, Younger Dryas; AL, Allerød; SMO, standard mean ocean water; PDB, Pee Dee Belemnite. $\delta^{18}\text{O} = [(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}}] - 1$. The transition from the Younger Dryas to the Preboreal is illustrated by a definite reduction in *Juniperus* and *Artemisia* pollen frequencies, reflecting the gradual retreat of open shrub-herb communities. A distinct rise in *Populus tremula* type coincident with the appearance of *Ulmus* pollen at the transition, followed by a large maximum in *Betula*, correspond to an expansion of forest communities responding to a change of climate. A more comprehensive discussion of available pollen data is given elsewhere^{7,39}. The rapid expansion of forests and formation of low-redox-potential soils is also reflected in the abrupt increase in content of iron, which could more easily be leached from soils under more reductive conditions. The decrease in sedimentation rate (with no significant change in annual accumulation of CaCO_3) and the decrease in the content of aluminium (not shown) suggests a limitation of erosion at that time. The development of the alga *Tetraedron* minimum in the lake at the Younger Dryas/Preboreal transition indicates rising summer temperatures³⁹. The main changes in vegetation are synchronous with those of $\delta^{18}\text{O}$ of authigenic carbonates. The scale of the calendar age (± 120 years) is based on a wiggle-match of AMS radiocarbon dates to the German oak chronology. The record of sedimentation rate has been smoothed.

☆ Deceased.

Because of natural variations in atmospheric ^{14}C concentration, the calibration curve showing the relationship between radiocarbon and calendar age is not linear. One of its most prominent features is the plateau at the radiocarbon age of $\sim 10,000$ ^{14}C yr BP (ref. 2), being also the generally accepted date of the Pleistocene/Holocene or Younger Dryas/Preboreal boundary³. It complicates an absolute dating and synchronization of that boundary between different sites when the timescale is reconstructed by radiocarbon dates. But even for the best absolute chronologies currently available (central Greenland ice cores and German tree-ring series), a significant discrepancy exists between estimates of the age of the boundary^{4,6}. This discrepancy can be interpreted either as a real delay in climate warming in Europe with respect to the North Atlantic or as a result of an as yet undetected error in counting annual ice layers and/or an incorrect match between German pine and oak chronologies². Another possibility of synchronization of that boundary is given by direct comparison of its position with respect to the worldwide synchronous ^{14}C plateau. This cannot be done in ice cores, but is possible in varved lacustrine sediments.

The archive analysed was the sediment of Lake Gościąg in central Poland⁷. The lake ($52^\circ 30' \text{N}$, $19^\circ 20' \text{E}$) contains two basins (24.6 m and 12.5 m), separated by a shallowing to ~ 6 m. The sediment is annually laminated, consisting of layers dominated by authigenic calcite and deposited during summer months, and layers rich in organic matter, deposited in the autumn/winter seasons. The varve-to-varve correlation of cores from both basins enabled the construction of continuous chronology containing $9,662 \pm 90$ varves for the lower part of the sediment. Because of the poor quality of the lamination in the upper part, this chronology is considered floating.

The abrupt climate changes at the Late Glacial/Holocene transition left a distinct signature in the sediments of Gościąg Lake (Fig. 1). The fluctuations in ^{18}O isotope composition of authigenic carbonates deposited in the lake coincide with major changes in the vegetation cover in the area⁷. The Gościąg $\delta^{18}\text{O}$ record ($\delta^{18}\text{O}$ is defined in Fig. 1 legend) closely resembles those observed in GRIP and GISP2 ice cores from central Greenland^{5,6} (compare Figs. 1 and 3). The Younger Dryas/Preboreal transition in the Gościąg sediments is marked by $\sim 2\text{‰}$ increase in $\delta^{18}\text{O}$, completed in ~ 70 years. Whereas a significant part of $\delta^{18}\text{O}$ signal in ice cores is probably related to changes of sources of moisture over central Greenland⁸, the prevailing influence of the westerly circulation maintained over the Europe during the glacial period⁹ makes the interpretation of our $\delta^{18}\text{O}$ record meaningful in terms of temperature change.

The accelerator mass spectrometry radiocarbon dating was done using plant macrofossils of terrestrial origin. The suite of dates (Fig. 2) enabled synchronization of the Lake Gościąg chronology with the German oak (younger) and German pine (older) chronologies², using the 'wigggle-matching' procedure¹⁰. Matching the set of youngest Gościąg samples to German oak chronology, we date the Younger Dryas/Preboreal boundary to $11,440 \pm 120$ BP. Thus, within the indicated error, this transition in central Europe was synchronous with that recorded in Greenland ice cores ($11,550 \pm 90$ BP, ref. 5; $11,640 \pm 250$ BP, ref. 11).

The early Holocene dates of analysed macrofossils (Fig. 2) clearly show a shift of Lake Gościąg varve chronology with respect to that of German pines. The wigggle-match of Gościąg to pine ^{14}C dates is excellent, enabling precise synchronization of both chronologies. The ^{14}C ages of two plateaux shown by our data agree very well with that reconstructed in high-precision dendrocalibration, indicating that systematic errors in dating and/or problems related to rebedding and contamination of Gościąg macrofossils are negligible. But the synchronization requires either a 'compression' of Lake Gościąg varve chronology or the revision of the tentative dendro-match between German oak and pine chronologies. The rejection of 200 varves from the 800-year sequence between 9,700 and 10,500 BP seems unlikely because the chronology is replicated in several cores

from two separate lake basins. The revision of the dendro-match, on the other hand, would expand the plateau of 8,900 ^{14}C BP to ~ 600 years. In fact, such a long plateau is suggested by data from Soppensee¹², where five dates of 8,900–9,000 ^{14}C BP occur in a time span of 700 years.

Independently of the absolute age, we can determine the position of the Younger Dryas/Preboreal boundary at the 10,000 ^{14}C BP plateau. The warming in Poland, as abrupt as in Greenland (Fig. 3), began about 250 years before the end of the plateau. On the other hand, the boundary in German pines, defined as the beginning of slow rises of $\delta^{13}\text{C}$ and δD in wood cellulose⁴, coincides with the end of the plateau. There is no doubt that the changes of isotopic composition in German wood started ~ 200 years after the main $\delta^{18}\text{O}$ increase in Lake Gościąg, during a period of distinct development of elm trees in Poland, that is, after the Younger Dryas period. Because both regions are situated only 1,000 km apart, exposed to the prevailing westerly circulation, it is very unlikely that the major warming began 200 years earlier in the region further east. Moreover, the increase in $\delta^{13}\text{C}$ and δD in trees continued for ~ 500 years, contrary to a very fast change observed in both Gościąg and ice core $\delta^{18}\text{O}$ data. We therefore conclude that the increase of $\delta^{13}\text{C}$ and δD in German pines is delayed somewhat and transformed with respect to the abrupt climate warming at the beginning of the Holocene.

The profiles of $\delta^{13}\text{C}$ and δD were derived from pine trunks excavated from Danube valley deposits⁴. Major warming at the

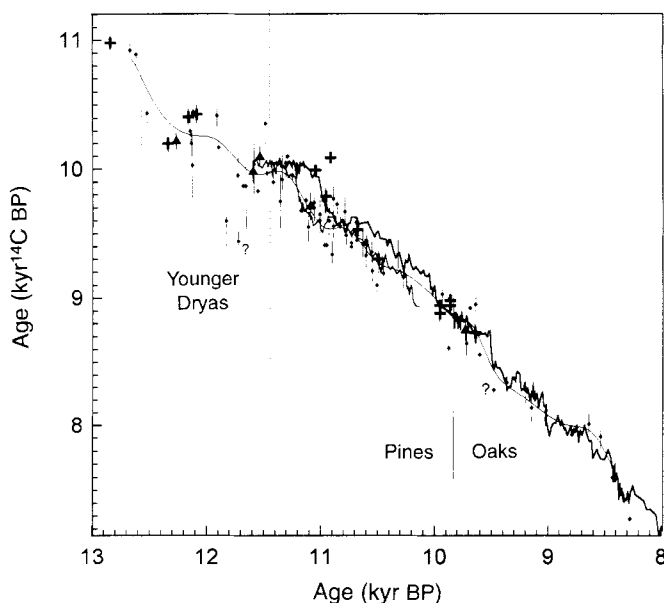


FIG. 2 AMS radiocarbon dates of terrestrial macrofossils extracted from the Lake Gościąg sediments, superimposed on the radiocarbon calibration data. The ages of terrestrial macrofossils are free of hard water or reservoir effect. To obtain enough material for dating (~ 1 mg), the macrofossils were collected from five precisely correlated cores. Apart from typical short-living macrofossils widely used in dating lake sediment (birch nutlets, birch fruit scales, bud scales and willow leaves), fragments of pine peridermis were collected. The suitability of peridermis fragments for dating sediments was checked by separate dating of pine peridermis and bud scales found in the same layers. The agreement of dates of paired scales and peridermis is satisfactory. Circles, Lake Gościąg macrofossils; triangles, Barbados corals¹⁴, crosses, Huon Peninsula corals¹⁵; heavy and light broken lines, German pines in original position², and shifted by 200 years, respectively. The calendar time-scale of Gościąg points (± 120 years) is based on a ^{14}C wigggle-match to German oaks. The smoothed curve is a cubic spline fitted to the Gościąg dates. The two samples indicated by question marks were probably contaminated.

Younger Dryas/Preboreal boundary must have lead to a marked influx of isotopically depleted water from the melting of Alpine glaciers to the Danube river and its flood plains. Even today, the Danube river is depleted in deuterium by about 10–15‰ during summer, owing to meltwater from the Alps (D. Rank, personal communication). As the δD of wood cellulose is mainly controlled by isotopic composition of environmental water¹³, the apparent 200-year delay of the analysed pines in response to an improving climate could result from enhanced contribution of isotopically depleted meltwater. The subsequent gradual increase of δD may then reflect a diminishing contribution of this source, when compared with local precipitation. The delay of $\delta^{13}C$ of wood cellulose to major warming is not well understood, but, it might result from a combination of the effects of increased water supply and the biological response to increased temperature.

In the late Allerød and Younger Dryas, the radiocarbon dates of Gościąg macrofossils (Fig. 2) generally confirm the U/Th calibration^{14,16}, showing a plateau at 10,400 ^{14}C BP and a rapid decline of ^{14}C age at the beginning of the Younger Dryas. The wiggles of the radiocarbon calibration curve reflect the changes of ^{14}C concentration in the atmosphere. Our data (Fig. 4a) confirm the large increase of $\Delta^{14}C$ at the onset of the Younger Dryas, by 40–70‰ within 300 years. ($\Delta^{14}C$ is defined in Fig. 4 legend.) This increase, demonstrated by four data points determined in three independent laboratories on two completely different types of material, would then be the largest known in the Late Glacial and Holocene records of atmospheric ^{14}C

concentration. On the other hand, the decrease in $\Delta^{14}C$ by 50–70‰ at the Younger Dryas/Preboreal boundary, is the largest and most rapid of declines occurring regularly between 12.3 and 9.8 kyr BP. The striking coincidence in distinct climate and radiocarbon variations suggests the involvement of some common causes.

Among possible causes of atmospheric $\Delta^{14}C$ variations are the changes of production rate of ^{14}C and reorganizations in the global carbon cycle. It is commonly accepted that the medium-term variations are connected with changing solar activity, whereas the secular $\Delta^{14}C$ trend in the Holocene reflects changes in the geomagnetic field¹⁷. The past geomagnetic variations have been reconstructed with a relatively good time resolution¹⁸. We used these data to simulate variations of atmospheric ^{14}C concentration. We ran simple box models^{19,20} with all parameters kept constant except for ^{14}C production rate, dependent on magnetic field according to Lal²¹. The results given by different models were very similar. In Fig. 4b we present the residuals between the smoothed curve of the observed $\Delta^{14}C$ and averages of those calculated from geomagnetically induced production rates.

The results of the calculations are sensitive to the choice of initial conditions and the model adjustment of the absolute value of ^{14}C production. In the original adjustment, the ^{14}C production rate corresponding to the current geomagnetic field maintains the steady-state $\Delta^{14}C = 0‰$ in the preindustrial atmosphere. The simulated curve that best fits the early Holocene data was obtained with an initial steady state of $\Delta^{14}C = 230‰$ and a production rate systematically lowered by 5%. Consequently the variability in geomagnetic field is then a good candidate for explaining the long-term $\Delta^{14}C$ decrease. In contrast, because of the buffering effect of the ocean, the concentration of atmospheric radiocarbon is not sensitive to hypothetical short-term variations of dipole moment in the range allowed by uncertainties in palaeomagnetic reconstructions. Moreover, the presence of an undetected magnetic anomaly concomitant with a major climate change seems very unlikely. We thus conclude that the geomagnetic Late Glacial/Early Holocene rapid shifts of atmospheric $\Delta^{14}C$ cannot be explained only by changes of the Earth's magnetic field.

The other mechanism might be changes in solar activity, although there is no direct evidence of this. But the regular occurrence of 180–220-year-wide $\Delta^{14}C$ maxima over the Holocene indicates that they could be induced by solar events similar to those responsible for the Spörer and Maunder minima¹⁷. Therefore, unless contradictory evidence is shown, we can only assume that the Late Glacial solar-wind-induced ^{14}C variations were similar to those observed in the Holocene. Although the amplitude of observed $\Delta^{14}C$ variations during the Late Glacial is similar, the duration (1000–2500 years) is distinctly larger than those attributed to the Sun in the Holocene²².

The general property of the global carbon cycle is that nearly all radiocarbon, which is produced in the atmosphere, decays in the deep ocean. For that reason, the concentration of radiocarbon in atmosphere depends on the size of the atmospheric reservoir and the rate of carbon exchange with the ocean. The large increase in atmospheric CO_2 concentration by 80 p.p.m. between 17 and 10 kyr BP is well documented²³, a change that might decrease the $\Delta^{14}C$ by 25–75‰ (refs 24, 25). The rise in CO_2 concentration could then explain the 20‰ decrease between the mean pre-Younger Dryas and Holocene $\Delta^{14}C$ residuals (Fig. 4b), but it is too slow to be responsible for the $\Delta^{14}C$ maximum within the Younger Dryas.

It is also accepted²² that the winds were stronger in glacial time and the Younger Dryas²⁶, and the enhanced rate of carbon exchange at the atmosphere–ocean interface²² could decrease $\Delta^{14}C$ by 20‰. But that mechanism works in the opposite direction and cannot explain the observed $\Delta^{14}C$ variations.

The last possible mechanism is the exchange with the deep oceans. This is especially important because a significant part of total gas exchange is related to the formation of North Atlantic

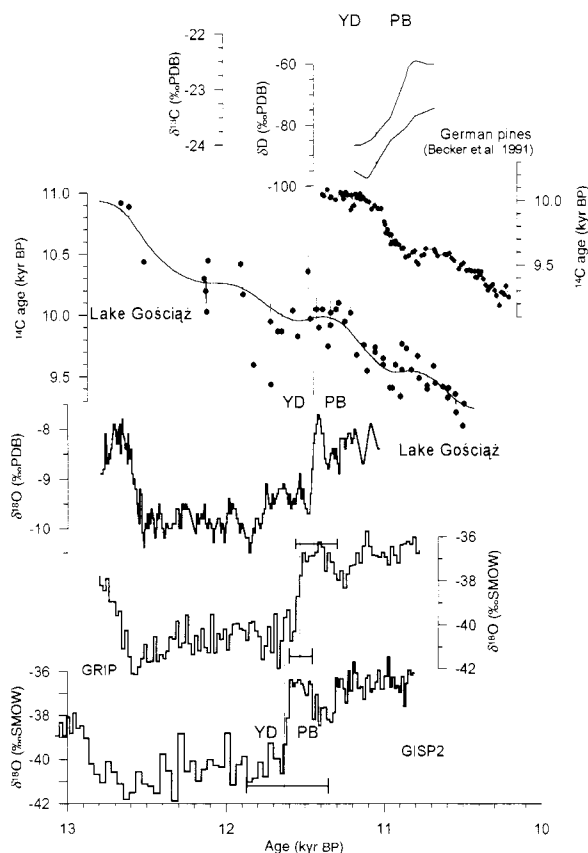


FIG. 3 Synchronization of the ^{14}C calibration curve and stable-isotope records representing the Late Glacial and Early Holocene. YD, Younger Dryas; PB, Preboreal. The match of Lake Gościąg and German pine chronologies (from ref. 4) requires a shift of one of chronologies by ~200 years. The changes in $\delta^{13}C$ and δD of German pines at the YD/PB boundary⁴ are represented by trend curves. Horizontal bars at the YD/PB transitions in Gościąg and Greenland ice represent age uncertainties ($\pm 1\sigma$).

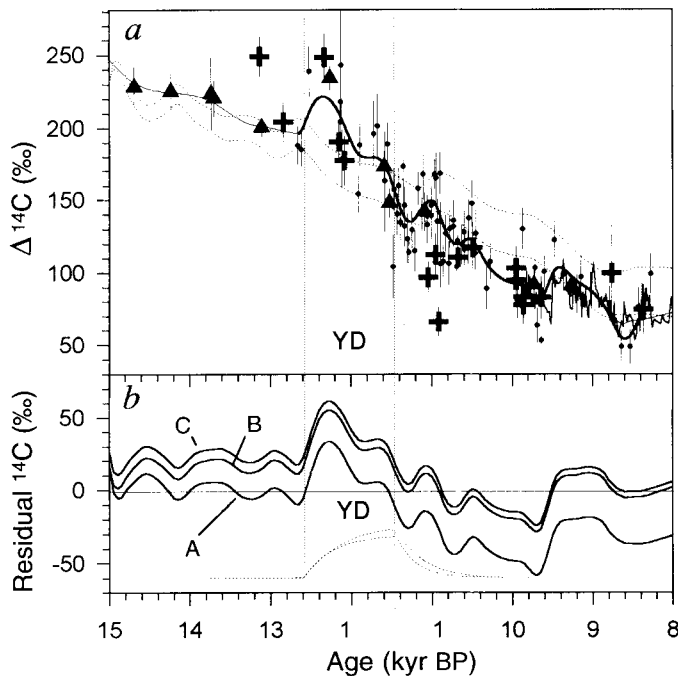


FIG. 4 *a*, Reconstruction of atmospheric ^{14}C concentration in the Late Glacial and Early Holocene. $\Delta^{14}\text{C}$ is defined as the relative deviation of radiocarbon concentration from that in the standard of the modern biosphere, and is expressed in per mil. The symbols are the same as in the previous figures. For clarity, the calibration data based on German pines are not included. The uncertainties in $\Delta^{14}\text{C}$ values resulting from error in the calendar age of the Gościąg varve chronology are neglected. The cubic splines fitted to Gościąg data (heavy smooth line) and to the coral data (thin smooth line), put together, serve as input data for model calculations (see text). The two broken lines represent $\Delta^{14}\text{C}$ curves, calculated on the basis of geomagnetically induced production rates: upper curve, $\Delta^{14}\text{C}_0 = 230\text{‰}$, ^{14}C production rate from Tric *et al.*¹⁸; lower curve, production rate systematically lowered by 5%. *b*, Differences between the reconstructed ^{14}C concentrations (data from Gościąg macrofossils and corals) and that calculated on the basis of geomagnetically induced production rates. A, $\Delta^{14}\text{C}_0 = 230\text{‰}$, ^{14}C production rate from Tric *et al.*¹⁸; B, production rate systematically lowered by 5%; C, $\Delta^{14}\text{C}_0$ lowered by 10‰. The lower broken line shows the amplitude of atmospheric $\Delta^{14}\text{C}$ change, simulated by the PANDORA box model²⁰, by changing the NADW flux from 30 Sv to 10 Sv and the Pacific loop from 15 Sv to 20 Sv and back, at the onset and termination of the Younger Dryas (YD), respectively. The upper broken line shows the amplitude of atmospheric $\Delta^{14}\text{C}$ change, simulated by the 13-box model³⁶, by changing the NADW flux from 20 Sv to 10 Sv and back, at the onset and termination of the YD, respectively.

Deep Water (NADW), and switching NADW formation 'on' and 'off' would be a plausible mechanism of abrupt climate changes²⁷. Many palaeoceanographic reconstructions^{28–31} give firm evidence for decreased NADW formation during both the Younger Dryas and the Late Glacial Maximum (LGM). That point has been challenged by two recent studies of benthic $\delta^{13}\text{C}$ (refs 32, 33). One hypothesis to explain the discrepancies relies on a shutdown of NADW transport to the deep ocean, but with transport to intermediate depths persisting during the LGM and the Younger Dryas³⁴.

The earlier ^{14}C dating of contemporaneous benthic and planktonic foraminifera suggested that the overall ventilation of the deep oceans at the LGM was much weaker than today³⁵. More recent data²⁰ suggest that the age of NADW during the LGM was twice as great as it is today, but the age of Pacific deep water was greater only by 70 ± 105 years. To simulate the corresponding change in atmospheric $\Delta^{14}\text{C}$ we used the standard PANDORA²⁰ and a 13-box model³⁶. By reducing the NADW flux by a factor of three and slightly increasing the circulation in the Indo-Pacific, the deep Atlantic becomes twice as old, the age of the deep Pacific increases by 150 years, and the atmospheric $\Delta^{14}\text{C}$ increases by about 30‰, in reasonable agreement with the observed amplitude of atmospheric $\Delta^{14}\text{C}$ changes at the boundaries of the Younger Dryas (Fig. 4*b*). A very similar $\Delta^{14}\text{C}$ wiggle (35‰) is obtained when the NADW flux is simply halved.

The $\Delta^{14}\text{C}$ record within the Younger Dryas, suggests that the lowest oceanic ventilation is in the earlier part of the Younger Dryas. This seems to be consistent with the amelioration of climate in the second half of the Younger Dryas, reconstructed in Lake Gościąg sediment⁷ and other regions in Europe³⁷. The rapid increase in atmospheric $\Delta^{14}\text{C}$ requires a decrease in deep-ocean ventilation in the early stage of the Younger Dryas that is significantly larger than the two-fold decrease in NADW flux alone. Unfortunately, available benthic/planktonic data are too scarce to reconstruct directly the magnitude of ventilation change in the Younger Dryas and, for the moment, cannot give a clear view of what actually occurred in the ocean during that period.

Recently it has been pointed out³⁸ that the possibility that the thermohaline circulation was shut down during the Younger Dryas, though tempting, could be eliminated by studying atmos-

pheric $^{14}\text{C}/^{12}\text{C}$ ratios. The abnormally high ^{14}C levels during the Younger Dryas shown by our results do not contradict the hypothesis that the thermohaline circulation decreased during the Younger Dryas. □

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A new family of monotremes from the Cretaceous of Australia

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AUSTRALIA'S second Mesozoic mammal, *Kollikodon ritchiei* (Monotremata, Kollikodontidae, new family) has an extreme bunodont molar morphology. The existence of two distinctively different families of monotremes in the Early Cretaceous suggests that the order originated long before the Cretaceous and was very diverse in at least the Australian portion of eastern Gondwana. With four families now known from Australia, it is probable that monotremes originated and diversified in the Australian/Antarctic sector of Gondwana, followed by a single dispersal (ornithorhynchid) to the South American sector before or during the early Paleocene. It is probable that kollikodontids are the sister-group of a steropodontid/ornithorhynchid/tachyglossid clade. *K. ritchiei* and *Steropodon galmani* are among the largest Mesozoic mammals known.

In 1971 the discovery of two mid-Tertiary teeth initiated a series of discoveries of toothed monotremes^{1–7}. Until now all monotreme dentitions were of a basically similar type and all were tentatively referred to the Ornithorhynchidae. This specimen (Fig. 1) is the third interpreted as mammalian from the Early Cretaceous deposits of Lightning Ridge, New South Wales^{3,8}.

Class Mammalia
Order Monotremata
Family Kollikodontidae nov.

Etymology. *Kollix*, Greek for a bun or roll; *odon*, Greek for tooth. The allusion is to the shape of the molars which resemble hot cross buns.

Type genus. *Kollikodon* nov.

Family diagnosis. That of the species *K. ritchiei* nov.

Kollikodon gen. nov.

Type species. *Kollikodon ritchiei* nov.

Generic diagnosis. That of the type species.

Kollikodon ritchiei gen. et sp. nov.

Etymology. The species name is in honour of Alex Ritchie, whose persistence in persuading the opal miners of Lightning Ridge to send fossils to a museum resulted in the recovery of the holotype.

Holotype. Australian Museum Palaeontological Collection F96602, a right dentary fragment with M_1 3 and alveoli for P_1 2 and M_4 , collected by Bob Sutherland.

Type locality and geology. $29^{\circ}28'12'' \pm 2''$; $147^{\circ}45'59'' \pm 2''$. Claim 30226, Moonshine area of the Coocoran opal field, at a depth of 15 metres. The sediment is a soft whitish clay interbedded with clay-ball conglomerates approximately 20–30 cm below a red sandstone which caps the opal-bearing clays throughout the Coocoran field. The Wallangulla Sandstone member of the Griman Creek Formation probably represents estuarine deposits.

Associated taxa and local fauna. This taxon forms part of the Lightning Ridge Local Fauna. The holotype and only known specimen was obtained from the same claim and horizon as a carnosaur tooth, turtle-shell fragment, unidentified vertebrae and a ?dinosaur phalanx. From the same Local Fauna elsewhere

at Lightning Ridge have come⁹ lungfish, teleosts, turtles, crocodiles, plesiosaurs, ornithischian and saurischian dinosaurs and the monotreme *Steropodon galmani*³.

Age. Early Cretaceous (Middle Albian)⁹.

Type species. *Kollikodon ritchiei* sp. nov.

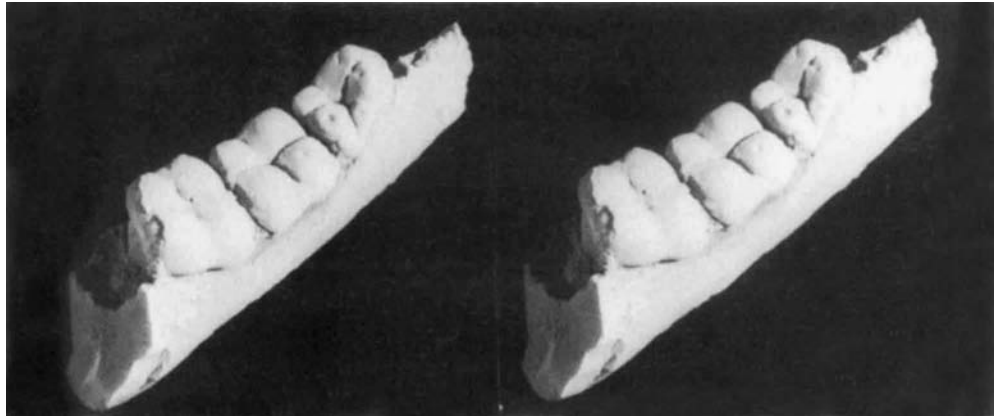
Species diagnosis. *Kollikodon ritchiei* differs from all other monotremes in having: at least four molars; bunodont molar morphology; no loph or vertical crests of any kind; no 'paraconids' on any of the molars; M_1 'protoconid' that is smaller than the 'metaconid'; markedly concave (upward in lateral view) tooththrow and curved dentary below M_1 4; very large mediobuccal cingular capsules; M_1 that is markedly smaller than M_2 ; reduced or absent posterior cingula on M_1 2; and near square rather than rectangular posterior molars.

The abrupt junction in size and form of alveolar/tooth morphology within the cheek-tooth dentition of *K. ritchiei* appears to represent the molar/premolar boundary. Anterior to this point, as in *Obdurodon dicksoni*, four alveoli appear, on the basis of their alveolar margins, to represent two double-rooted premolars, P_{1-2} . Posterior to this point, the three molariform teeth and alveolar remnants posterior to these represent M_1 4. Because the dentary is broken at the level of M_4 , it is not possible to determine if there were more than four molars. Although other monotremes have two premolars either as dental anlage or well-formed teeth (*Ornithorhynchus*, *Obdurodon*), none of these have more than three molars^{1,4,5,7,10}. Considering molar number in other pre-tribosphenid mammals¹¹, the higher number is almost certainly a plesiomorphic feature within the order.

The two roots of P_2 suggest a crown length of ~2.8 mm. Other measurements are: M_1 length, 5.7 mm; anterior width, 3.5 mm; posterior width, 4.1 mm. M_2 length, 5.6 mm; anterior width, 5.4 mm; posterior width, 5.3 mm. M_3 length, 5.5 mm; anterior width, 5.7 mm; posterior width, 5.2 mm. Below P_2 the dentary is 6.1 mm deep and ~3.6 mm wide (the dentary being damaged here). At this point, the dental canal is 2.1 mm in diameter.

The conclusion that *Kollikodon ritchiei* is a monotreme depends on dental morphology. Although its teeth cannot be directly compared with those of tachyglossids, all of which are edentulous, or the living platypus *Ornithorhynchus anatinus*, which are vestigial and ephemeral¹², there are other toothed monotremes. The Miocene *Obdurodon dicksoni*^{4,5}, known from a skull that is clearly ornithorhynchid in structure, has a well-formed dentition with complex molars. These in turn share an array of features with the molars of *Steropodon galmani*. Shared features that, in combination, can be regarded as distinctively monotreme include an anteroposteriorly compressed M_1 trigonid that lacks a paraconid (autapomorphic among Mesozoic mammals), high transverse loph-like trigonid and talonid blades (autapomorphic), very large talonid (autapomorphic among pre-tribosphenid¹³ mammals), prominent anterior, posterior and buccal cingula (?symplesiomorphic in mammals), abrupt discontinuity in size between the small P_2 (as indicated by alveoli) and large M_1 (autapomorphic among all Mesozoic mammals) and wide talonids without entoconids (autapomorphic among Mesozoic mammals). The lack of entoconids (and hence presumably protocones in the corresponding upper molars) indicates the pre-tribosphenid position of monotremes^{4,5} and convergence in talonid enlargement. Because all of these features, except the transverse, loph-like blades, are also present in *K. ritchiei*, this taxon is concluded to also be a monotreme. *K. ritchiei* and *S. galmani* further share very large size compared with other Mesozoic mammals and a large dental canal (which suggests a need for relatively extensive innervation and blood supply at the front of the head, as in modern platypuses which have sensitive rhinaria and electrosensory organs). In *K. ritchiei* the dentary narrows markedly anterior to the position of M_1 , as it does in *O. anatinus*. As in all other monotremes, there is no evidence of a canine alveolus although the specimen is missing much of the anterior portion of the dentary. The teeth have very shallow

FIG. 1 Stereo view of holotype of *Kollikodon ritchiei* gen. et sp. nov. in posterobuccal view. Magnification, $\times 2$.



roots in contrast to those of *S. galmani*, and the premolar (and possibly molar) alveoli invade the canal space, as they do in at least some ornithorhynchids including species of *Obdurodon* and *Ornithorhynchus*.

In contrast to the teeth of other monotremes, those of *K. ritchiei* are unique in lacking vertical cutting blades. All cusps are low, rounded and domed. There are remnants of prominent buccal cingula with associated cingular cusps on at least M_1 – 2 . Although the primary cusps present may be protoconids (anterobuccal), metaconids (anterolingual), hypoconids (posterobuccal) and hypoconulids (posterolingual), cusp homologies are uncertain because of doubts about the therian affinities of monotremes⁵. The tips of several cusps have breached enamel which could assist horizontal shearing. However, there are no indications that thegosis¹⁴ maintained these as secondary, horizontal cutting blades. Evidence for thegosis in other monotremes is also missing⁴, and it is possible that tooth 'sharpness' in the group, whether vertical or horizontal, was structural only and not maintained, by deliberate wear of one tooth against another, throughout life. The extreme horizontally distributed wear

exhibited by the paratype of *Obdurodon insignis*² and holotype of *Monotrematum sudamericanum*⁷ may support this hypothesis.

Within the Order Monotremata, loss of the fourth and sometimes third molars in ornithorhynchids (for example, *Monotrematum*, *Obdurodon* and *Ornithorhynchus*) suggests they are monophyletic and more derived in this regard than kollikodontids, which retain at least four molars. Although less persuasive, loss of teeth in all tachyglossids (*Tachyglossus*, *Zaglossus*, *Megalibgwilia*) would suggest they are the sister-group of ornithorhynchids, which also exhibit tooth loss, rather than the sister-group of kollikodontids which do not. DNA hybridization studies suggest¹⁵ that tachyglossids and ornithorhynchids diverged in the early Tertiary or Late Cretaceous, which further suggests that these two families are monophyletic and together the sister-group of the Early Cretaceous *Steropodon galmani*. Although *S. galmani* was originally described as a possible ornithorhynchid³, given the phylogenetic understanding expressed in Fig. 2, Ornithorhynchidae would be paraphyletic. The solution is to recognize the family-level distinction of *S. galmani*. Steropodontidae fam. nov. (type genus *Steropodon*; containing only *S. galmani*) may be diagnosed as monotremes that: differ from tachyglossids in having teeth; differ from ornithorhynchids in having relatively unreduced M_3 , long-rooted molars, deep mandibles and in lacking pseudoentoconids; and differ from kollikodontids in lacking M_4 and in having high-crowned molars with well-developed transverse blades.

A second hypothesis of relationships that ignores the molecular data would be that *Kollikodon ritchiei* is the sister-group to tachyglossids. However, there are no synapomorphies that would support this view. Even if there were, its retention of a well-formed and differentiated dentition would distinguish it at a family level from undoubted tachyglossids, all of which have lost even dental anlagen¹⁶.

The presence of four families of monotremes in the Australian sector of Gondwana suggests an origin and diversification within the Australian (or Australian/Antarctic) sector with one dispersal (by early Paleocene time) of ornithorhynchids^{6,7} to the South American sector. That the first two Mesozoic mammals known from Australia represent distinctively different families of monotremes suggests first that monotremes may have dominated the Early Cretaceous mammal assemblages of at least the Australian portion of Eastern Gondwana in contrast to the South American portion¹⁷. It also demonstrates a greater breadth for the monotreme radiation than previously understood which, considering the age of the Lightning Ridge monotremes, suggests that this order probably had a time of origin no later than the Jurassic. Distinctive features of the monotreme dentition such as the lack of a 'paraconid' on M_1 indicate that the basic monotreme molar structure is not therian-like, and that any other similarities between monotremes and therians may be convergent⁵. Conversely, increased understanding about monotreme dental morphology fails to clarify the enigmatic interordinal relationships

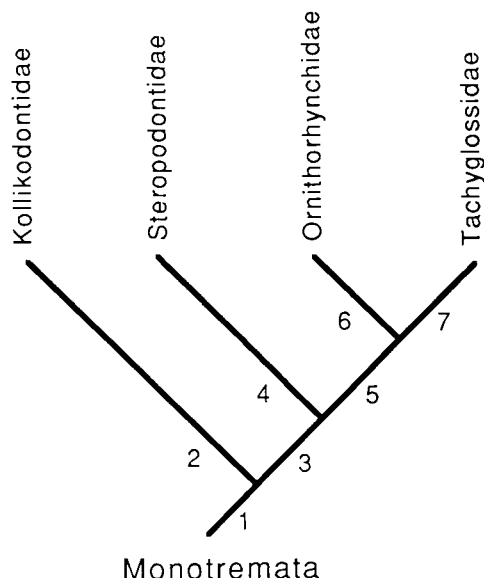


FIG. 2 Cladogram of intraordinal family relationships that incorporates molecular indications of a late Cretaceous/early Tertiary common ancestry for ornithorhynchids and tachyglossids. Apomorphies mainly for lower dentitions (common denominator for all taxa): 1, paraconid not developed on M_1 ; extremely abrupt premolar/molar junction, very wide talonid, high and transverse molar blades; 2, bunodont molars, loss of transverse molar blades, loss of all paraconids, protoconid smaller than metaconid on M_1 ; 3, loss of M_4 ; 4, deep dentary; 5, extreme reduction of M_3 ; 6, pseudoentoconids developed; 7, complete loss of all teeth.

of the group⁵. The large mandibular canal suggests the possibility that kollikodontids, like other monotremes, had a bill and/or electrosensory organs at the front of the face. The large size of both *K. ritchiei* and *S. galmani*, which are among the largest Mesozoic mammals known, may reflect the cold conditions of southeastern Australia in the early Cretaceous¹⁸.

Kollikodon ritchiei was a platypus-size monotreme that fed on material that needed crushing but not shearing. Similar adaptations are evident in marine predators, such as sea otters, crabs and some fish that feed on hard-shelled animals. □

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Continual change in mate preferences

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SECONDARY sexual characters are highly variable both within¹ and between species^{2–6}. Closely related species often differ markedly in sexual morphology but hardly at all in non-sexual traits^{2–5}. Here we show that Fisher's runaway process of sexual selection is intrinsically unstable and naturally leads to continual change in sexual traits. Runaway leads to semi-stable exaggeration of female preference for a male sexual character, followed by a slow decay of both traits until runaway is triggered again in a different direction. The process then repeats itself resulting in continual change in male sexual traits through time. Allopatric populations are thus expected to diverge without drift or substantial changes in selective pressures. If there is significant mutation bias acting on the male trait, continual change stops and a stable equilibrium appears. Such an outcome is more likely when exaggeration of the male sexual trait signals good genes.

Let t be a male trait used by females in mate choice and p be the strength of female preference. We assume that both t and p

are sex-limited and have a polygenic basis. The per generation change in the mean values caused by Fisher's runaway process is

$$\begin{pmatrix} \Delta \bar{t} \\ \Delta \bar{p} \end{pmatrix} = \frac{1}{2} \begin{pmatrix} G_t & B \\ B & G_p \end{pmatrix} \begin{pmatrix} \beta_t \\ \beta_p \end{pmatrix} + \begin{pmatrix} -u \\ 0 \end{pmatrix} \quad (1)$$

where G_t and G_p are additive genetic variances of t and p , respectively, B is the additive genetic covariance between these two traits, and u measures any biased mutation acting on the male trait⁷. The effect of selection is given by selection gradients (β_p, β_t) evaluated at the population means using an assumption of weak selection⁸

$$\beta_p = \frac{\partial}{\partial p} \ln W_f, \quad \beta_t = \frac{\partial}{\partial t} \ln W_m, \\ W_f = \exp(-bp^2), \quad W_m = \exp(a\bar{p}(t - \bar{t}) - ct^4) \quad (2)$$

where W_f and W_m are individual female and male fitness, respectively. Female fitness is determined by the strength of preference. We assume that female costs are minimized when there is no discrimination ($p=0$) and increase symmetrically with the strength of preference at a rate b . Male fitness is the product of mating success and survivorship. Mating success is determined by female mate preference. Mean female preference can be either for males with larger ($\bar{p}>0$) or smaller ($\bar{p}<0$) than average trait values⁷. The optimal expression of the male trait under natural selection occurs when $t=0$. Unlike previous models^{7,9}, male survival is assumed to decrease with the fourth power rather than a quadratic. This is more in line with Fisher's¹⁰ original assumption that the cost of the male trait is very small around the optimum but increases very quickly beyond a certain limit. The coefficients a and c calibrate these two selective effects.

Figure 1 shows an evolutionary trajectory generated by the model. The population starts near the origin, where females show no preference ($\bar{p}=0$) and males have a trait value that achieves maximum survivorship ($\bar{t}=0$). It quickly evolves away from the origin in a runaway process^{9,10}. The system converges to a curve, on which evolutionary change slows down, but does not stop completely. Here the male trait is exaggerated and females show corresponding strong preference. Female preference then slowly declines until runaway starts again but this time in a negative direction. This negative runaway stops when the system converges on another semi-stable branch at which t and p are negatively exaggerated. Then a very slow movement in a positive direction starts until runaway is triggered toward positive values.

The evolutionary trajectory can be studied further by decomposition into fast and slow dynamics^{11,12}. Runaway, or the fast dynamics, is given by neglecting the cost to female mate preference b and mutation bias u that are assumed to be much smaller than the other parameters^{11,12}

$$\Delta \bar{p} = \frac{B}{2} (a\bar{p} - 4c\bar{t}^3), \\ \Delta \bar{t} = \frac{G_t}{2} (a\bar{p} - 4c\bar{t}^3). \quad (3)$$

Setting $\Delta \bar{p} = \Delta \bar{t} = 0$ yields a curve of the equilibria for the fast dynamics (red line in Fig. 1). We can distinguish a stable (solid) and unstable (dashed) part of the curve^{9,13,14}. Points B and D are the two points where the slope of the curve of equilibria is the same as the slope of the fast dynamics trajectory, $\Delta \bar{p}/\Delta \bar{t}$. The subarc between B and D is unstable and the two branches outside are stable.

Evolution near the curve of equilibria is given by the slow dynamics which includes the effects of b and u . The per generation change in mate preference is (see appendix 2 of ref. 11)

$$\Delta \bar{p} = \frac{G_p}{2} (-2b\bar{p} + ua). \quad (4)$$

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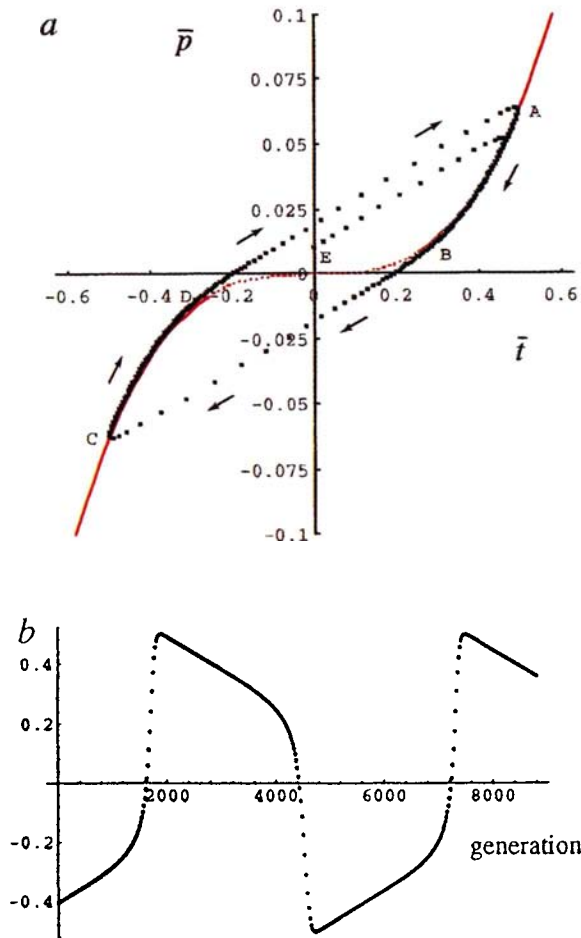


FIG. 1 Cyclic evolution caused by Fisher's runaway process is plotted as *a*, the joint evolution of the mean female preference ($\bar{p}$) and mean male trait ($\bar{t}$) and *b*, as change in the mean male trait ($\bar{t}$) per generation. The population is plotted every 20 generations. As in previous models^{11,12} we simplify the dynamics by a decomposition into fast and slow dynamics. This is justified on the assumption that mutation bias u and the cost to female mate preference b are both much smaller than the other parameters. We also make a weak selection assumption that the fitness coefficients a and c are both smaller than the additive genetic variances G_t and G_p which are of order unity (that is, $b, u \ll a, c \leq G_t, G_p \approx O(1)$). We make the simplifying assumption of constant genetic variance. Under these conditions, the additive genetic covariance between the male trait and female preference converges quickly to $B = aG_tG_p/2$ (ref. 11). The fast dynamics (ignoring b and u) define a curve of the equilibria (red line) $\bar{p} = 4c\bar{t}^3/a$, which is unstable (dashed line) when its slope $12c\bar{t}^2/a$ is less than that of the evolutionary trajectory during runaway $B/G_t = aG_p/2$, but otherwise stable (solid line). Once close to the curve of equilibria, the evolutionary trajectory is given by the slow dynamics (including b and u) which causes movement along the curve of equilibria. The points A and B define the transition between runaway and the slow dynamics ($\bar{p}_A, \bar{t}_A = (2a^2G_p^{3/2}/3\sqrt{6c}, aG_p^{1/2}/\sqrt{6c})$ and $(\bar{p}_B, \bar{t}_B) = (a^2G_p^{3/2}/2\sqrt{6c}, aG_p^{1/2}/2\sqrt{6c})$). As the model is symmetrical, points C and D are found by changing the sign of A and B. In this simulation, mutation bias was set to zero, so the equilibrium point E lies at the origin and is unstable. Parameter values are $a=0.4, b=0.001, c=0.05, G_t=0.5, G_p=0.5, u=0$.

Change in the male trait is now purely a function of the mean preference and follows $\dot{\bar{t}} \approx (a\bar{p}/4c)^{1/3}$. These equations describe movement along the stable branches of the equilibrium curve towards equilibrium at point E

$$(\bar{p}_E, \bar{t}_E) = \left(\frac{ua}{2b}, \left(\frac{ua^2}{8bc} \right)^{1/3} \right). \quad (5)$$

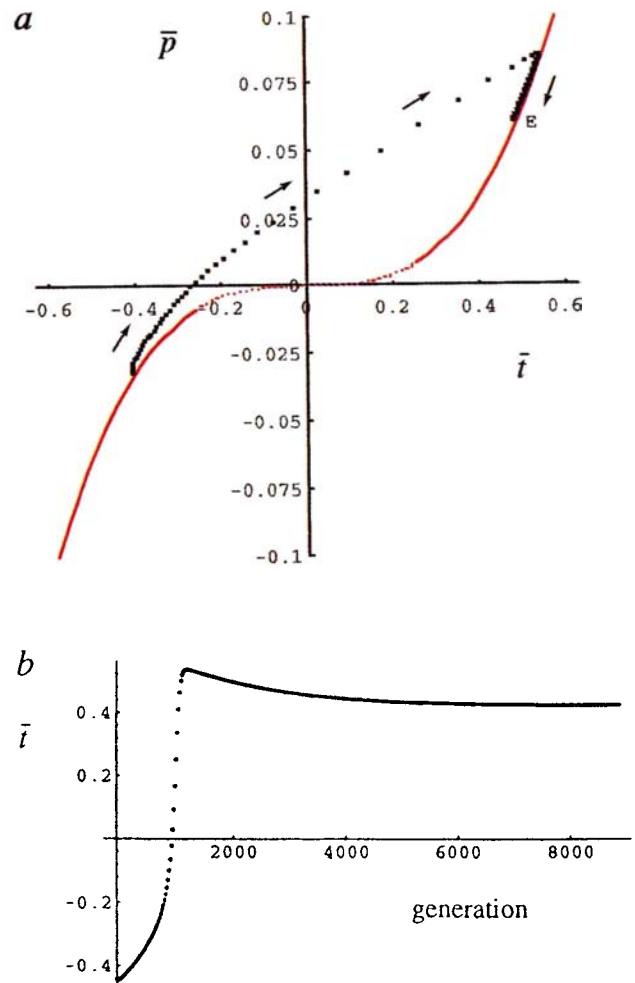


FIG. 2 Stable evolution caused by Fisher's runaway process. In this example mutation bias on the male trait u is large enough and the cost of choice b small enough for $\bar{p}_E > ua/2b$. So the equilibrium point E lies on the stable arc of the equilibrium curve and is globally stable. Parameter values are $a=0.4, b=0.001, c=0.05, G_t=0.5, G_p=0.5, u=0.001$.

Stability depends on whether E lies on a stable branch of the equilibrium curve. If $\bar{p}_E > \bar{p}_B$, the population smoothly converges on the equilibrium which is globally stable (Fig. 2). But if $\bar{p}_E < \bar{p}_B$, then the fast dynamics takes the population away from the equilibrium once it reaches point B and the equilibrium E is never reached (Fig. 1). This conforms to the Poincaré-Bendixon theorem that a two-dimensional autonomous system with a single unstable equilibrium has a cyclic trajectory if the system is confined to a finite area¹⁵.

If mutation bias is negligibly small ($u=0$), which is likely to be the case in many sexual traits, there is an unstable equilibrium at the origin. The male trait cycles between positive and negative values and the time between successive runaways derived from equation (4) is $\tau \propto 1/bG_p$. As the cost to female mate preference b is assumed to be small, male traits can persist for many generations. The time period during which a runaway occurs is very much shorter than τ and is unaffected by b (see equation (3)).

If u is positive, stability depends on a number of other parameters. Of particular importance is the cost of mate preference b . If b is large compared to u , the equilibrium is unstable. The population traces the same trajectory as when $u=0$. Cyclic behaviour is seen so long as b remains greater

than the critical value

$$b_c = \frac{6u\sqrt{6c}}{aG_p^{3/2}} \quad (6)$$

A further conclusion concerns the magnitude of additive genetic variance. Greater additive variance in female mate preference G_p increases exaggeration, the rate of turnover and stability but has no effect on the location of equilibrium. In contrast, additive variance in the male trait G_r has little impact. It neither affects exaggeration, rate of turnover, stability or the location of equilibrium. G_r only affects the speed of Fisher's runaway process, which occurs in such a short time interval as to be instantaneous in comparison to the interval between successive runaways.

Cyclic evolution is a general property of Fisher's runaway process. The only change we have made to the standard model⁷⁻⁹ is to make selection on the male trait weak around the natural selection optimum allowing runaway to be initiated and to decrease survival chances rapidly beyond a certain value. We note that strong stabilizing selection at the origin will inhibit runaway but continual evolution follows once runaway occurs⁷⁻⁹. Similar instability can be generated in models of the handicap process in which the male trait acts as an indicator of heritable viability, so-called good genes (Y.I., T. Kubo and A.P., manuscript in preparation). But a stable equilibrium is a more common outcome. Mutation bias is known to be strong on viability traits^{16,17} and the handicap model assumes that viability traits strongly influence the expression of the male sexual trait^{8,12}. As we have seen significant mutation bias is necessary for a stable, non-zero equilibrium.

Current explanations of the diversity of sexually selected traits have stressed the importance of random genetic drift^{9,18} and distinct selective pressures in geographically separated populations¹⁹. Given cyclic evolution, such slight differences affecting selection or genetic parameters (that is, a , b , c , u , G_r or G_p) will cause allopatric populations to fall out of phase quickly and evolve distinct sexual phenotypes. The consequences of this are more easily visualized by increasing the dimensionality of the model to multiple female preferences for different male traits (such as colours, songs or feathers)¹¹, which greatly increases the number of possible semi-stable states (A.P. and Y.I., manuscript in preparation). The instability of Fisher's runaway process may well explain the high variability of sexual traits between closely related species²⁻⁶. □

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Ejaculate quality and the success of extra-pair copulations in the zebra finch

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IN many passerine birds, sperm competition^{1,2} is intense and extra-pair paternity frequent³. The outcome of sperm competition is often determined by relative sperm numbers^{4,5}, and theory predicts that males should maximize the number of sperm they ejaculate during extra-pair copulations^{6,7}. Differences in sperm quality between males also affect the outcome of sperm competition⁴. Here we report that the swimming velocity of sperm of the zebra finch, *Taeniopygia guttata*, varies predictably within males, and is determined, together with sperm numbers, by the time since last ejaculation. By performing extra-pair copulations outside their own-pair copulation period, males maximize both the quality and number of sperm in ejaculates. These effects are a consequence of the way sperm are stored and mature in the male reproductive tract. The disproportionate success of extra-pair copulations⁸, also seen in other birds⁹, may therefore be explained in terms of the independent effects of sperm numbers and velocity.

When male zebra finches that were rested (that is, had not copulated in the previous 7 days) ejaculated several times in rapid succession into model females¹⁰, the quality of their ejaculates decreased markedly and their sperm store, the seminal glomera¹¹, became depleted. The average path velocity of sperm, measured using computer-aided sperm analysis (Hobson Sperm Tracker), from two ejaculates one hour apart from each of 13 rested males fell by almost half, from 32.88 ± 4.40 (s.e.) $\mu\text{m s}^{-1}$ to $18.75 \pm 1.95 \mu\text{m s}^{-1}$ (paired $t_{12} = 3.074$, $P < 0.01$). Five males that ejaculated a mean of 3.20 ± 0.37 times to exhaustion transferred a mean total of $8.05 \times 10^6 \pm 2.95 \times 10^6$ sperm in successively smaller ejaculates (first, $5.29 \times 10^6 \pm 1.56 \times 10^6$; second, $1.91 \times 10^6 \pm 1.17 \times 10^6$; third, $0.75 \times 10^6 \pm 0.44 \times 10^6$; fourth, $0.166 \times 10^6 \pm 0.08 \times 10^6$; two-way analysis of variance (ANOVA) on $\log(x+1)$ sperm numbers: male, $F_{4,10} = 7.62$, $P < 0.005$; ejaculate number, $F_{3,10} = 13.91$, $P < 0.001$). A mean of $1.32 \times 10^6 \pm 0.56 \times 10^6$ sperm (16% of the original number, $8.05 \times 10^6 + 1.32 \times 10^6$) was left in their seminal glomera. Thus 84% of the sperm in the seminal glomera of rested males was available for ejaculation. Of the remainder, $79.46\% \pm 8.50$ sperm were morphologically non-normal (senescent) compared with those in ejaculates ($32.27\% \pm 8.20$; paired t -test on arcsin transformed values, $t_4 = 4.41$, $P < 0.02$).

The mechanism responsible for this difference in ejaculate quality was a gradation of sperm quality within the seminal glomera: the velocity of motile sperm ($P < 0.005$), the proportion of motile sperm ($P < 0.03$), and the proportion of morphologically normal sperm ($P < 0.05$) all increased from the most proximal region to that nearest the cloaca (Fig. 1). The velocity of sperm from the cloacal ($33.44 \pm 4.89 \mu\text{m s}^{-1}$) and mid region ($17.44 \pm 2.09 \mu\text{m s}^{-1}$) of the seminal glomera did not differ significantly from that in first and second ejaculates, ($t_{21} = 0.084$, $P > 0.1$; $t_{21} = 0.457$, $P > 0.1$, respectively). This gradation of sperm quality is presumably due to a combination of maturation, as in the mammalian epididymis^{12,13}, and a sorting process.

The temporal consequences of these differences in ejaculate quality were determined by measuring the rate of sperm production (Fig. 2). As the seminal glomera of rested males contain 8.05×10^6 sperm available for ejaculation (above and ref. 14), and sperm are produced at a rate of 1.885×10^6 per day (Fig. 2), recovery of both sperm number and velocity after depletion requires 4-5 days.

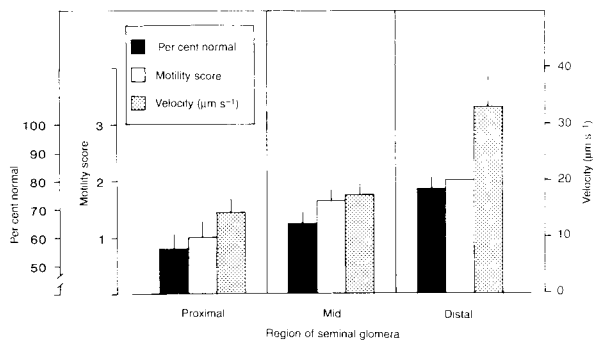


FIG. 1 Differences in the percentage of morphologically normal (black bars), percentage of motile sperm and average path velocity of motile sperm (grey bars) in three regions of the zebra finch seminal glomera: proximal (nearest the ductus deferens), mid, and distal (nearest the cloaca); error bars show s.e. Sperm motility and velocity were measured in a medium containing 80% phosphate buffered saline (with 1% bovine serum albumin), 10% skimmed milk and 10% semen at 35 °C. The differences between regions are significant. Percentage of normal sperm: analysis on arcsin transformed data, two-way ANOVA, there was a significant effect of region ($F_{2,8} = 5.87$, $P < 0.03$) and bird ($F_{4,8} = 13.76$, $P < 0.002$). Percentage of motile sperm (scored as 0, 1 or 2, from low to high): Kruskal-Wallis analysis of variance, $H = 7.48$, $P = 0.024$. Velocity: a two-way ANOVA showed a significant effect of region ($F_{2,8} = 12.53$, $P < 0.005$), but no bird effect ($F_{4,8} = 2.35$, $P = 0.141$).

Differences in sperm velocity between zebra finch ejaculates result from the way males store sperm, but the evolutionary consequences are considerable. Models of sperm competition^{6,7} predict that males should ejaculate more sperm during extra-pair copulations than during pair copulations and extra-pair copulations should thus have a disproportionately high fertilization success. Our results confirm both predictions. First, as in many birds⁵, male zebra finches perform extra-pair copulations after their own-pair copulation period has ended¹⁵. This can result in an extra-pair ejaculate containing over seven times as many sperm as a regular pair ejaculate (Fig. 2a). Extra-pair ejaculate size in the zebra finch is therefore controlled through the behavioural timing of copulations¹⁶, rather than by any physiological ability to adjust sperm numbers, as occurs in the taxa¹⁷. Second, sperm competition experiments in the zebra finch⁸ show that a single extra-pair copulation can be disproportionately successful and fertilize between 54 and 84% of eggs, apparently as a result of the greater number of sperm¹⁸. However, because motility and fertilization success of ejaculated sperm are positively associated¹⁹, the results presented here indicate here that this effect could be due to sperm number, sperm quality or both. In mammals, sperm velocity is positively associated with fertilization success (H. D. M. Moore, personal communication), but the mechanism is unknown. Whether

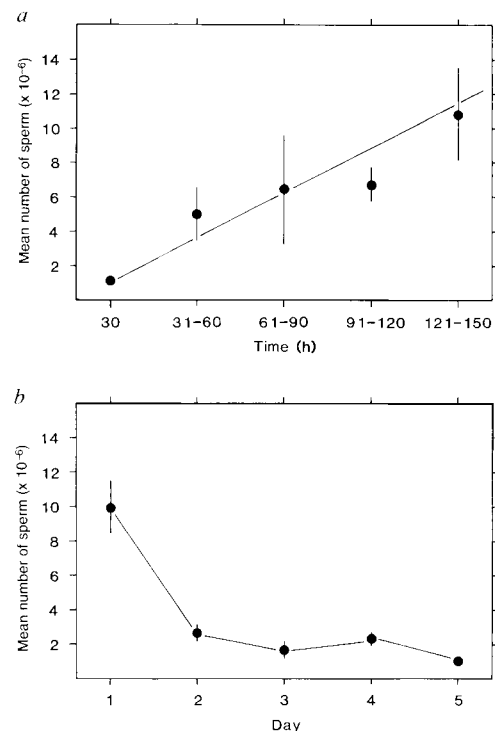


FIG. 2 Daily sperm production rate in the zebra finch was estimated in two independent ways. *a*, Males were depleted to exhaustion (3 ejaculates followed by 2 'refusals' to copulate, all within 3 h) and allowed to copulate with a model female. Each of 10 males provided 4 or 5 ejaculates (following depletion each time) spread fairly evenly over a 6-day period. An ANCOVA showed no effect of bird ($F_{9,23} = 0.414$, $P = 0.915$), but a significant effect of time ($F_{1,23} = 44.07$, $P < 0.001$), on sperm numbers. The graph shows the relationship between time since last ejaculation and number of sperm per ejaculate. After combining data across males (sperm numbers = $0.075 \text{ time (h)} + 0.340$; $P < 0.001$; $r^2 = 0.362$), the slope of this relationship indicates that males produce sperm at a rate of 1.80×10^6 per day. *b*, Ten different rested males copulated twice each day with model females over 5 successive days. Sperm numbers are determined by time (two-way ANOVA, $F_{4,29} = 19.28$, $P < 0.001$), but not bird ($F_{9,29} = 1.43$, $P > 0.2$). After excluding the data for day 1, that is, removing the rested male effect, no significant heterogeneity existed ($F_{3,28} = 1.116$, $P = 0.359$) and the daily sperm output was 1.97×10^6 per day. The mean of the two methods (1.80×10^6 and 1.97×10^6) was 1.885×10^6 sperm per day.

sperm velocity affects fertilization is unknown in birds, but high-velocity sperm may be more likely to enter the fertilization set by their rapid passage through the vaginal region, which is extremely hostile to sperm^{14,20}. The disproportionate fertilization success of extra-pair copulations recorded in some species^{3,9,21} may result from greater numbers of faster moving sperm. □

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Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors

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DOPAMINERGIC neuronal pathways arise from mesencephalic nuclei and project axons to the striatum, cortex, limbic system and hypothalamus^{1,2}. Through these pathways dopamine affects many physiological functions, such as the control of coordinated movement and hormone secretion³. Here we have studied the physiological involvement of the dopamine D2 receptors in dopaminergic transmission, using homologous recombination to generate D2-receptor-deficient mice. Absence of D2 receptors leads to animals that are akinetic and bradykinetic in behavioural tests, and which show significantly reduced spontaneous movements. This phenotype presents analogies with symptoms characteristic of Parkinson's disease^{4,5}. Our study shows that D2 receptors have a key role in the dopaminergic control of nervous function. These mice have therapeutic potential as a model for investigating and correcting dysfunctions of the dopaminergic system.

A targeting vector was constructed to transfect embryonic stem (ES) cells that contained a dopamine D2 receptor (*D2*)

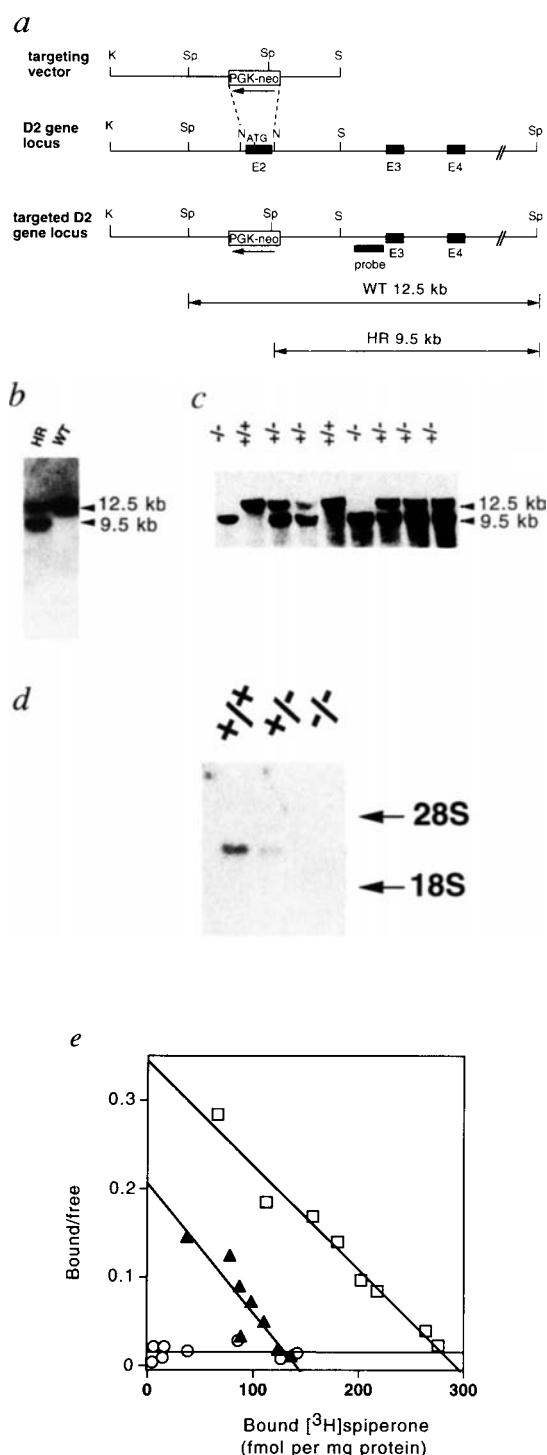


FIG. 1 Disruption of the D2-receptor gene. a, D2-receptor knockout strategy. Top, the PGK-neo cassette was used to disrupt the dopamine D2 gene coding frame. Middle, schematic representation of the targeted region of the D2 gene locus. Bottom, the mutated D2 gene locus. The thick black bar indicates the genomic 1.4-kilobase (kb) *PstI* probe, used to identify recombinants. The sizes of the *SpeI* fragment from wild-type and mutated alleles are indicated. Restriction enzyme sites: K, *KpnI*; Sp, *SpeI*; S, *SalI*; N, *NcoI*. b, Genomic Southern blot analysis of DNA from electroporated ES cells. Arrows indicate the presence of the 9.5-kb fragment of the mutant allele (HR) compared with the wild-type (WT) 12.5-kb fragment. c, Genomic Southern blot analysis of mouse tail DNA. The mouse genotypes are depicted on top of each lane: +/+, wild type; +/-, heterozygote; -/-, homozygote. d, Northern blot analysis of dopamine D2 receptor expression in total RNA extracted from striata of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) D2-deficient mice. e, Scatchard analysis of the saturation isotherm for [³H]spiperone binding to striatal membranes from +/+ (□), +/- (▲) and -/- (○) D2-receptor deficient mice. The results shown are representative of three independent experiments each conducted in duplicate. The dissociation constants (mean±s.e.) were 25.5±1.7 pM for wild-type mice (□), 17.6±3 pM for heterozygous mice (▲) and inestimable for homozygous mice (○). The maximum binding capacities were 290±20 fmol per mg protein for wild-type mice and 135±19 fmol per mg protein for heterozygous mice.

METHODS. The dopamine D2-receptor gene was isolated from a mouse 129-ES/EMBL3 genomic library. A 6.5-kb *KpnI*-*SalI* genomic fragment corresponding to the D2 gene was isolated and subcloned into pBlueScript (Stratagene). From this clone, a 0.9-kb *NcoI* fragment, containing exon 2 and flanking intron sequences, was removed and replaced by a 1.4-kb segment of the PGK-neo cassette⁶, inserted in the opposite transcriptional orientation, relative to the D2 gene. This targeting vector was linearized and electroporated in P1-ES cells. ES cells carrying the disrupted D2 allele were revealed by Southern blot analysis using a random primed ³²P-labelled *PstI* probe on *SpeI* genomic digests. Positive clones were injected into C57BL/6 embryos at the blastocyst stage. Chimaeric offspring were mated with C57BL/6 mice.

Germline transmission of the mutant allele was assessed by Southern blot analysis of tail DNA isolated from the agouti-coat progeny. Northern blot analysis was performed with 10 µg of total RNA isolated from mice striata by a lithium chloride extraction method, using the 550-bp *SmaI*-*PvuII* fragment of the mouse D2 cDNA²⁷. D2-receptor ligand-binding assays were carried out with striatal tissue homogenized with a polytron in ice-cold 10 mM Tris-HCl pH 7.5 containing 5 mM EDTA. The homogenates were centrifuged at 19,000g for 40 min at 4 °C, then the supernatants were collected and centrifuged again. 15 µg of striatal membranes was used for ligand-binding assays with [³H]spiperone (specific activity 114 Ci mmol⁻¹; Amersham), as previously described^{28,29}. Concentrations of [³H]spiperone of 10–600 pM were used and nonspecific binding was determined in the presence of 1 µM (+)-butaclamol. Binding data were analysed with the EBDA-LIGAND program (Elsevier-BIOSOFT).

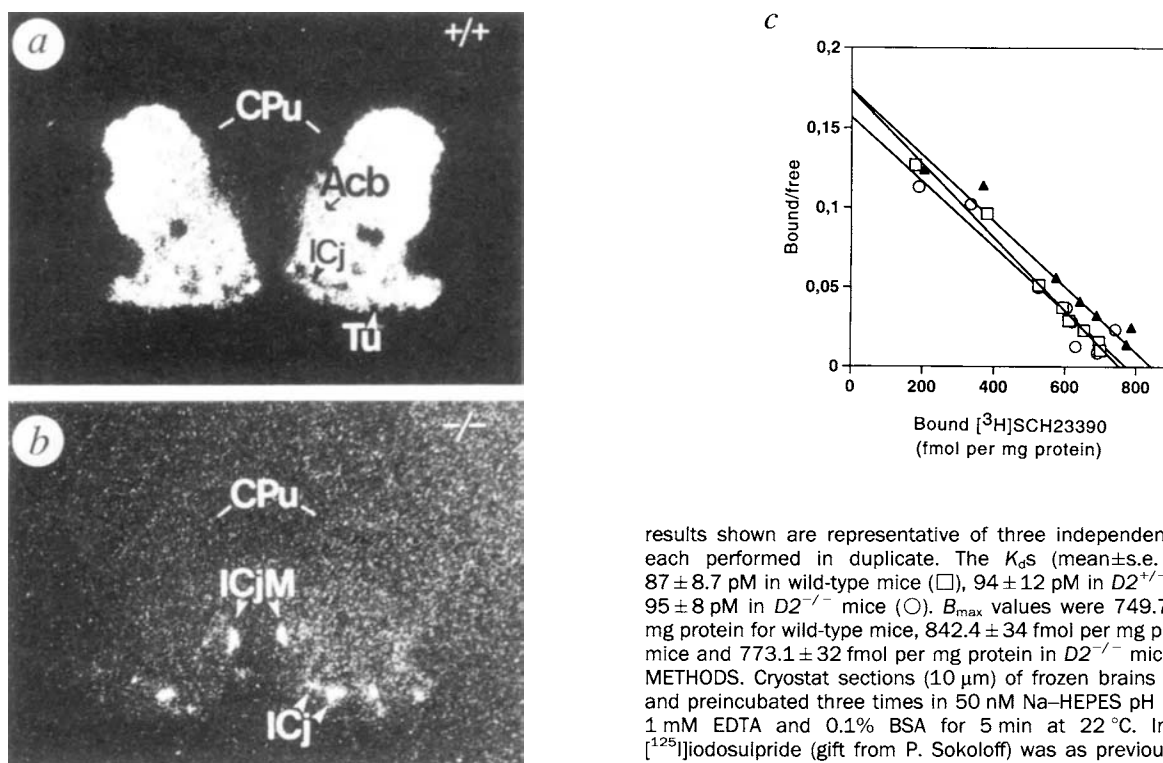


FIG. 2 $[^{125}I]$ iodosulpride autoradiographic localization and $[^3H]SCH23390$ binding. **a**, In wild-type animals $[^{125}I]$ iodosulpride reveals D2- and D3-receptor-binding sites. **b**, Only D3-receptor-binding sites are present in $D2^{-/-}$ mice. Abbreviations: Acb, nucleus accumbens; CPu, caudate putamen; ICj, islands of Calleja; ICjM, islands of Calleja major; Tu, olfactory tubercle. **c**, Scatchard analysis of the saturation isotherm for binding of the D1 dopamine receptor antagonist $[^3H]SCH23390$. The

genomic fragment in which exon 2 was deleted and replaced with a PGK-neomycin cassette⁶ (Fig. 1a). One positive ES clone was obtained (Fig. 1b) and used to establish mutant mice (Fig. 1c). Reduced fertility was observed in the breeding of homozygous $D2$ -deficient mice; litters could only be obtained by breeding homozygous mice with the heterozygous counterparts. Comparison of $D2$ -receptor-deficient mice with their normal littermates showed a 15% ($n=20$) reduction in body weight and a 10–15% reduction in water and food intake. In addition, a $0.7^\circ C$ ($n=7$, $P<0.001$) reduction in the body temperature of homozygous $D2$ -deficient mice was observed.

Northern analysis of RNA from the striata of heterozygous and homozygous $D2$ -deficient littermates confirmed reduced or absent expression of the $D2$ receptor (Fig. 1d). This result was supported by $[^3H]$ piperone isotherm saturation analysis of membrane preparations from striatal tissue. We observed a marked reduction in binding sites in heterozygous mice (maximum binding capacity $B_{max}=135\pm19$ fmol per mg protein) compared with wild type ($B_{max}=290\pm20$ fmol per mg protein), despite similar affinities for the ligand (dissociation constant $K_d=17.6\pm3$ and 25.5 ± 1.7 pM, respectively). $D2$ -binding sites were completely absent in the homozygous mouse (Fig. 1e).

In situ binding using $[^{125}I]$ iodosulpride, a ligand with high affinity for D2 and D3 receptors, showed strong labelling of the striatum and olfactory tubercles in a wild-type mouse (Fig. 2a). The weak labelling present in the $D2$ -deficient homozygotes showed that the binding in the wild type corresponded almost entirely to D2-binding sites. Interestingly, the residual labelling corresponds to the regions where D3 receptors are localized⁷ (Fig. 2b). These data also indicate that the expression of $D3$ is

not affected in the $D2$ null mice. Consistently, RNA analysis revealed no significant differences in the pattern of expression of the $D3$ and $D4$ genes in the $D2$ -deficient background (not shown).

Because dopamine D1 and D2 receptors have antagonistic effects on cyclic AMP levels, we investigated whether the absence of D2 receptors could be compensated for by changes in D1 receptor levels by *in vitro* binding analysis using a D1-receptor-specific ligand, $[^3H]SCH23390$. The Scatchard analysis of these studies revealed no significant differences either in the affinity for the ligand ($K_d=86.7\pm8.7$ ($+/+$), 94 ± 12 ($+/-$) 95 ± 8 pM ($-/-$)), or in the number of D1-receptor sites ($B_{max}=750\pm14$ ($+/+$), 842 ± 34 ($+/-$) and 773 ± 32 ($-/-$) fmol per mg protein) (Fig. 2c). These data show that the partial or complete absence of D2 receptors does not affect the expression of other members of the dopamine receptor family.

We next addressed the effect of the lack of D2 receptors at the level of gene expression. We performed northern blot analysis of total RNA and *in situ* hybridization of coronal brain sections from homozygous and control mice, using probes for enkephalin⁸, substance P⁹, dynorphin¹⁰, glutamic acid decarboxylase (GAD)¹¹ and tyrosine hydroxylase¹². The expression of the first three peptides is known to be influenced by the activity of D1 and D2 receptors. Enkephalin is coexpressed with the D2 receptor in the medium spiny neurons of the striatopallidal pathway¹³. Substance P and dynorphin are expressed in neurons of the striatonigral pathway that express D1 receptors¹³. All three peptides participate in the control of the firing of striatopallidal and striatonigral neurons; GAD¹⁴ and tyrosine hydroxylase¹², involved respectively in the synthesis of γ -amino-

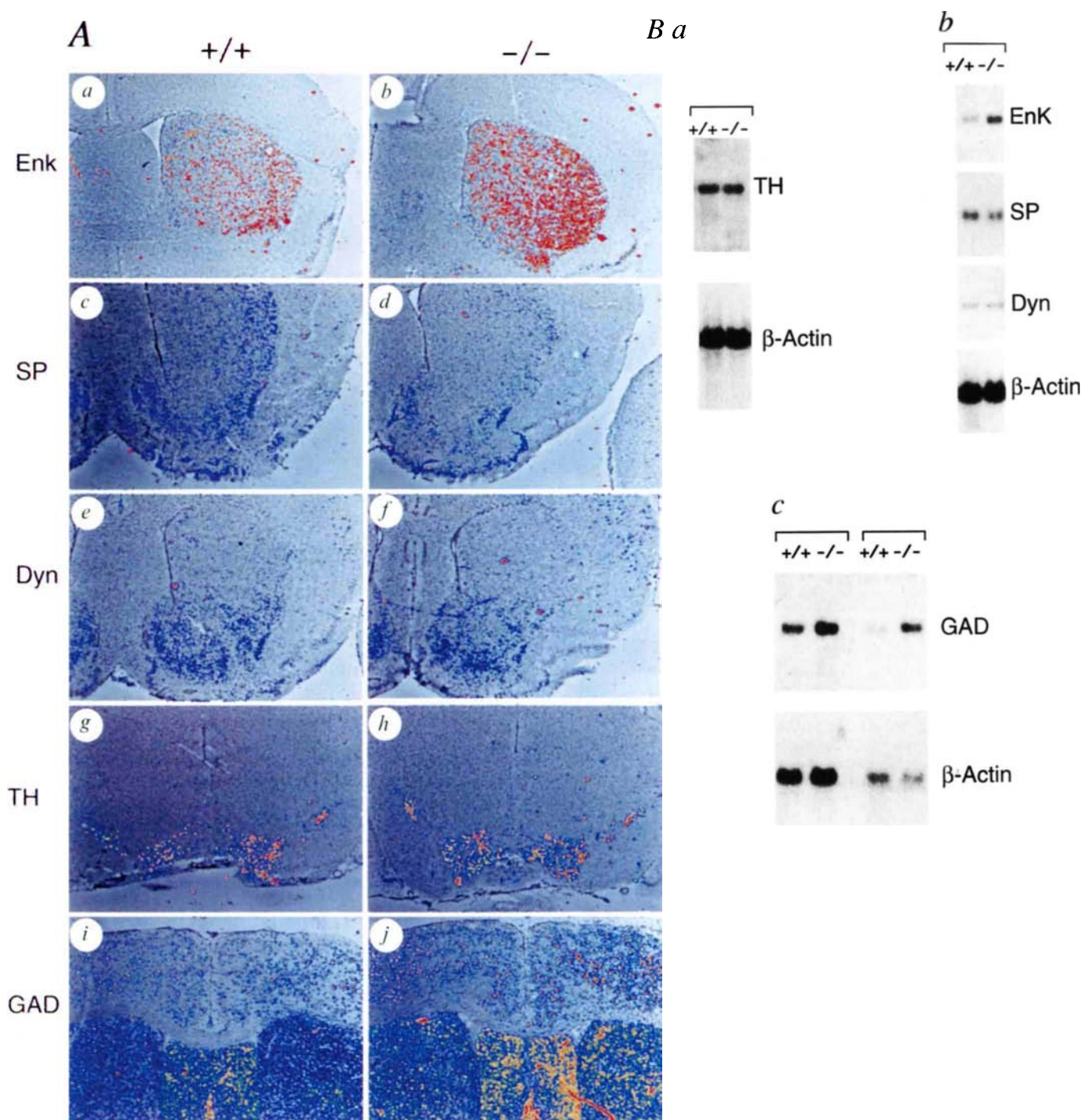
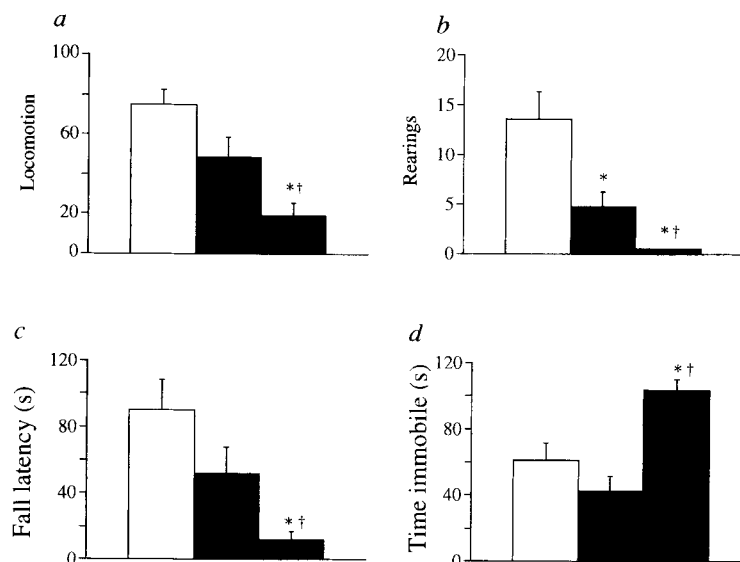


FIG. 3 mRNA analysis of neuropeptide expression in wild-type and mutant mouse brains. **A**, *In situ* hybridization using antisense RNA probes corresponding to enkephalin (Enk) (a, b), substance P (SP) (c, d), dynorphin (Dyn) (e, f), tyrosine hydroxylase (TH) (g, h) and glutamic acid decarboxylase (GAD) (i, j) were carried out in wild-type (+/+) and mutant (-/-) mice. **B**, Northern blot analysis of neuropeptide expression. 10 μ g of total RNA from different regions of the brain of control (+/+) and D2-deficient mice (-/-) ($n=3$) was analysed with different probes. a, Midbrain; b, striatum; and c, striatum (left) and cortex (right). Abbreviations for probes are as for A. β -Actin was used as internal control.

METHODS. For *in situ* hybridization, cryostat sections (10 μ m) were prepared and hybridized with the specific antisense RNA probes. Antisense RNA probes were prepared by standard conditions using T3, T7 or SP6 polymerases using [35 S]CTP. RNA probes were subjected to partial alkaline hydrolysis to reduce the average probe length to 150 nucleotides.

The mouse Enk probe was synthesized from a cDNA fragment of 762 bp (PvuII-XbaI)⁸. The 465-bp SP probe corresponds to the rat 5' portion of exon 7 (ref. 9). The Dyn probe used corresponds to the 1,700-bp PstI-EcoRI cDNA fragment subcloned into pSP64 (ref. 10). The TH probe corresponds to the 1,522-bp BamHI-KpnI cDNA fragments¹² and the GAD probe was synthesized from a 1,000-bp EcoRI-SalI cDNA fragment subcloned into pSP65 (ref. 11). Hybridizations were performed as described³⁰. All slides were exposed to Kodak NTB-2 autoradiography emulsion for 24 h (Enk, TH) or 1 week (SP, Dyn, GAD). For northern blot analysis equal amounts of RNA (10 μ g) were assessed by optical photodensitometry and orange acridine staining of RNA in the gel. β -Actin gene expression was analysed in each experiment and used as internal standard control to quantify the relative changes in the expression of each gene. Data were quantified with a Bio-Imaging Analyzer using Fuji imaging plates. These measurements showed the variation in the expression of each gene to be less than 15%.

FIG. 4 Motor behaviour of D2 knockout heterozygous and homozygous animals compared with wild-type animals. The behaviour of D2 knockout mice reared in isolated cages at least 1 week before the tests was evaluated in the open-field (a, b)¹⁷, rotarod (c)¹⁹ and ring (d)¹⁸ tests. □, Wild type; ▨, heterozygote; ■, homozygote. For the open-field test, each mouse was placed in the middle of a 30-cm-diameter enclosure, the floor of which was partitioned into 12 squares of equal surface area. Animal behaviour was recorded for 5 min with a video camera. Their cumulative locomotion (a, number of squares crossed) and rearings (b) were later counted from the video recordings ($n=10$, 5 and 5 for wild-type, heterozygous and homozygous mice, respectively). For the rotarod test, each mouse was placed on a 6-cm-diameter rod covered with rubber and left for 30 s to habituate to the rod without rotation. The rod was then set in motion with a rotation speed of 2 turns per min and the behaviour of the animals was video-recorded for a maximum of 2 min. Each mouse was given a maximum of three trials. The best performance (the time spent on the rod without falling, in s) was kept for analysis ($n=10$, 10 and 10 for wild-type, heterozygous and homozygous mice, respectively). For the ring test, each mouse was placed on a ring (5.5 cm diameter) attached to a stand 16 cm above the base²⁰ and behaviour was video-recorded for 3 min. The periods of immobility (absence of any voluntary movements) were later measured ($n=8$, 8 and 7 for wild-type, heterozygous and homozygous mice, respectively). All data are expressed as means $\pm$ s.e.m. and were compared between groups by a non-parametric analysis of variance for



unrelated samples (Kruskal Wallis). Comparisons between groups were then made using the Mann-Whitney test; *, $P<0.02$ compared with wild-type mice; †, $P<0.02$ heterozygous compared with homozygous mice.

butyric acid (GABA) and dopamine, are thus indicators of the synthesis of these neurotransmitters. The expression of enkephalin messenger RNA was increased by 40% in the striatum of D2-deficient mice (Fig. 3Aa, b and Bb). This finding is reminiscent of the increase in enkephalin seen in the striatum of 6-OHDA-lesioned animals¹⁵. In this respect, the absence of dopamine provoked in these animals parallels the effect obtained by the elimination of the D2 receptor. A 15% decrease was observed in substance P expression (Fig. 3Ac, d and Bb). In contrast, the level of expression of dynorphin seemed to be unaltered (Fig. 3Ae, f and Bb). Tyrosine hydroxylase expression was unchanged, suggesting that in D2-deficient mice endogenous dopamine levels are not altered (Fig. 3Ag, h and Ba).

Northern blot analysis showed an average 40% increase in GAD expression in the cortex and 20% in the striatum of knockout compared with normal mice (Fig. 3Bc). *In situ* hybridization supported these observations (Fig. 3Ai, j). Other regions expressing GAD showed no significant difference in the D2-receptor-deficient animals. Thus lack of D2 receptors has major consequences for specific gene expression in restricted areas.

Histological analyses of testes and ovaries of knockout mice showed that the size of these organs is greatly reduced, perhaps as a result of impaired function of the hypothalamus-pituitary-gonadal axis. D2 receptors are, in fact, also expressed in the anterior pituitary gland, where they exert a negative control over prolactin synthesis and release¹⁶. These data might implicate a role for prolactin in gonad development. Thus the inactivation of the D2 receptor has pleiotropic physiological consequences.

Given the importance of dopaminergic transmission in the coordination and regulation of movements, the behaviour of D2 null mice was tested. D2-deficient mice showed normal reflexes. However, initial observations indicated that these mice had slower movements than their normal littermates. Posture was abnormal, with fore and hind paws flattened to the ground. When allowed to walk on a flat surface, they exhibited an abnormal gait, with sprawled hind legs. This behaviour was quantified by carrying out general behavioural tests (open field¹⁷, ring test¹⁸ and rotarod¹⁹) on homozygous, heterozygous and wild-type male littermates. The comparative study revealed a motor impairment in homozygous mice, also evident to a lesser extent

in the heterozygous mice. Homozygous D2-deficient mice showed a marked reduction in locomotion (74%) and backward movements in the open-field test (Fig. 4a). Notably, they showed no vertical behaviour in this test (rearing; Fig. 4b).

The rotarod apparatus was used to test the ability of mice to coordinate movements. Homozygous mice spent significantly less time on this apparatus than heterozygous or wild-type mice (Fig. 4c). In addition, they showed a cataleptic-like behaviour as quantified in the ring test²⁰. Homozygous mice showed a significant increase in the time spent immobile compared with heterozygous and wild-type mice (Fig. 4d).

An understanding of the dopaminergic control of neural functions and hormone physiology is central to clarifying the function of dopamine in the nervous system. Dopamine has a key role in directing the control of movements and hormone secretion. At present, five different membrane receptors are known to be stimulated by dopamine²¹, although the contribution of each receptor to particular physiological functions remains unclear. The study of dopaminergic function by classical means, such as the treatment of live animals with dopamine-specific agonists or antagonists, has been greatly complicated by the lack of specificity of these drugs.

D2 and D1 receptors are the most abundant dopaminergic receptors expressed in the brain. The parallel activation by dopamine of these two receptors in the basal ganglia leads to the activation of two pathways whose synergistic action controls coordinated movements. Unexpectedly, the elimination of D1-receptor expression by homologous recombination, which would be predicted to affect the locomotion-enhancing direct pathway, leads instead either to no effect²² or to mild hyperlocomotion in homozygous mice²⁰. These results contradict pharmacological studies where the use of D1-receptor antagonists results in a cataleptic response in treated animals^{23–25}. In contrast, D2-deficient animals display a phenotype that broadly resembles D2 antagonist treatment^{3,26}, indicating that the effect observed by antagonist treatment is due to the specific blocking of D2 receptors and not other members of the dopamine receptor family.

The phenotypic changes observed in D2-receptor-deficient animals support an essential role for these receptors in the dopaminergic control of physiological functions. One can also

speculate that D2 receptors represent the core function of dopaminergic pathways related to motor control and that the other dopamine receptor subtypes might modulate its activity.

The increase in GAD expression may represent a compensatory mechanism in the brain of D2-receptor-deficient mice. In this situation, alteration of the normal circuitry might lead to increased firing of subthalamic neurons, resulting in an increased stimulation of nigral neurons, which would not be sufficiently balanced by the activity of striatonigral neurons. This could result in a distortion of the feedback signal to the cortex and to other components of the motor pathway. These altered functions might be compensated for in part by increasing GABA levels. Electrophysiological and functional studies must test this hypothesis.

The D2-deficient mouse phenotype strikingly resembles the lack of spontaneous movements, akinesia, abnormal gait and posture constituting the extrapyramidal symptoms of Parkinson's disease. Thus these mice can be used for molecular dissection of the altered physiological processes responsible for such behaviour, and so possibly lead to new treatments for these symptoms. Furthermore, the D2-deficient background will provide an *in vivo* system for testing drugs directed at D3, D4 or other dopamine receptors. □

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A P2X purinoceptor expressed by a subset of sensory neurons

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ATP is known to depolarize sensory neurons, and may play a role in nociceptor activation when released from damaged tissue^{1–10}. Here we report the molecular cloning and characterization of a new member of the P2X receptor family^{3,11,12}, P2X₃, expressed by these cells. The channel transcript was present in a subset of rat dorsal-root-ganglion sensory neurons, some of which express nociceptor-associated markers; it was absent in other tissues that were tested, including sympathetic, enteric and central nervous system neurons. Moreover, when expressed in *Xenopus* oocytes, the channel showed an ATP-dependent cation flux. P2X₃ is the only ligand-gated channel known to be expressed exclusively by a subset of sensory neurons. The remarkable selectivity of expression of the channel coupled with its sensory neuron-like pharmacology suggests that this channel may transduce ATP-evoked nociceptor activation.

We have isolated a dorsal-root-ganglion (DRG)-specific 3.8-kilobase transcript that encodes an open reading frame of 397 amino acids with substantial sequence homology to known P2X receptors (Fig. 1a)¹³. We used an *in vitro* coupled transcription-translation system and found that a protein of the appropriate relative molecular mass (*M_r*) of ~45K, which we named P2X₃, could be synthesized from the complementary DNA clone (Fig. 1b).

a	
DRG	1MNCI SDFFTYETTK SVVVKSWIIG IINRAVQLLI ISYFVGWVFL 50
PC12	MVRRLARG-W -A-WD---P- VI--RNRRL- FVH-M-----LL---WY---I
VAS	MARRLQDELS AF--E-D-PR M-L-RNKKV- V-F-LI--VV LV-VI---V
	TM-1
DRG	51 HEKAYQVRDT AIESSVVTKV KGFGRY.... .ANRVMVDSD YVTPQQTSTV 100
PC12	VQ-S--DSE- GP---II--- --ITMS.... .EDK-W--EE --K--E-G--
VAS	Y--G--TS.S DLI---SV-L --LAVTQLQG LGPQ-WD-A- --F-AH-D-S
DRG	101 FVITKIIVT ENQMQGFCPE ..NEEKYRCV SDSQCGPERF ..PGGGILTG 150
PC12	VS--R-E-- PS-TL-T--- SMRVHSST-H --DD-IAGQL DMQ-N--R--
VAS	--VM-NF--- PQ-T-H-A- N..PEGGI-Q D--G-TPGKA ERKAQ--R--
DRG	RCVN.YSSVL RTCEIQGWCP TE.VDTVEMP IM.MEAENFT IFIKNSIRFP 200
PC12	H--PY-HGDS K---VSA--- V..DG-SDNH FLGKM-P--- -L-----HY-
VAS	N--P-FNGTV K---F--- V-VD-KIPS- A-LR-----L-----S-
DRG	201 LFNFEKGNLL PNLTDKDIR CRFHPKAPF CPILRVGDVV KFAGQDFAKL 250
PC12	K-K-S---IA SQKSD.YL-H -T-DQSDS-Y ---F-L-FI- EK--EN-TE
VAS	R-KVNR--V EEVNGTYM-K -LY-KIQH-L --VFNL-Y-- RES---RS-
DRG	251 ARTGGVLGIK IGWVCDLDRK WDQCIPIKYSF TRLDGVSEKS SVSPGYNFRF 300
PC12	-HK---I-VI -N-N---LS ESE-N--- R--PKYD.. PA-S-----
VAS	-EK---V--T -D-K---WH VRH-K-I-- ..QFH-LYGEK NL---F---
DRG	301 AKYKMGNS EYRTLLKAFG TRFDLVYGN ACKFNIIPTI ISSVAAFTSV 350
PC12	----INGTT TT---I--Y-- -T-DQSDS-Y ---SL-----NLAT-L--I
VAS	-RHF.VQ--T NR-HLF-V-- -HF-I--D-K-- --D---M TTIGSGIGIF
	TM-2
DRG	351 GVGTVLCDDI LLNFKLGADH YKARKFEE..VTETTLKGTA 400
PC12	---SF---W- --T-MNKNL -SKH--DKVR TPKHPSSRPW --LALVL-NI
VAS	--A-----LL --HI-PKRHY --QK--K..YA-DMGP-EG
DRG	401 STNPVFASDQ ATVEKQSTDS GAYSIGH..... 450
PC12	PPP-SHY-ND NPPSPFP-GEG PTLGE-AELP LAVQSPRPCS ISALTEQVVD
VAS	EHD--AT-ST LQLQENMRTSL.....
DRG	451 481
PC12	TLGQHMGQR PVVPSPSQ DSTSTDLGLA L
VAS	

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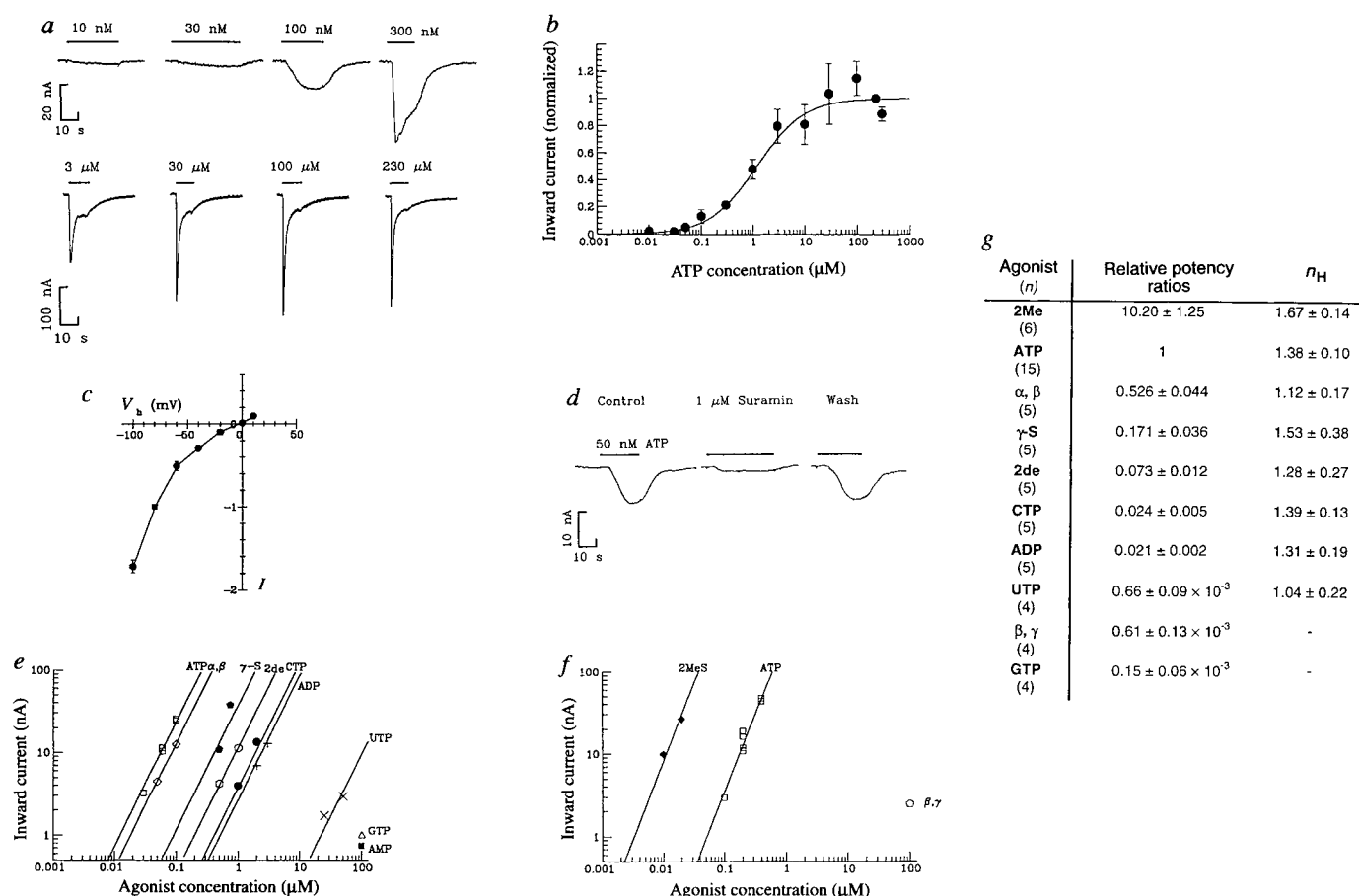


FIG. 2 Electrophysiological properties of P2X₃ expressed in *Xenopus* oocytes. **a**, Inward currents evoked by increasing concentrations of ATP in oocytes injected with P2X₃ complementary RNA. **b**, Concentration-response curve for ATP ($n=5$). Data from each oocyte were fitted simultaneously with the Hill equation (least-squares fit, with the constraint of a common EC₅₀) and normalized with respect to the fitted maximum in each set. **c**, Current-voltage relation of the P2X₃ response. Data are normalized to the response obtained at -80 mV (filled square; $n=4$). **d**, Effect of $1 \mu\text{M}$ suramin on the response to 50 nM ATP. Holding potential -80 mV. **e**, **f**, Dose-response curves for 2 oocytes used for the calculation of the potency ratios in **g**. Results were fitted simultaneously with Hill equations constrained to be parallel and to have a maximum fixed at a value much higher than the amplitude of the responses fitted. Errors were estimated with Fieller's theorem²². The fit was repeated without the constraint of parallelism, to obtain the Hill slope, n_H . Abbre-

viations: α, β , α, β -methylene-ATP; γ, δ , ATP- γ, δ ; 2de, 2'-deoxyATP; 2MeS, 2-methylthio-ATP; β, γ , β, γ -methylene-ATP.

METHODS. P2X₃-encoding plasmid DNA was digested to position -58 upstream of the initiator methionine (Promega Erase-a-base system). The plasmid was cut with *Xho*I and subcloned into a pSP64T derivative (pSP64T.clon) between *Sma*I and *Sal*I sites. The resulting plasmid was linearized with *Pst*I, and cRNA transcribed with SP6 polymerase¹⁵. cRNA (20–70 ng) was injected into *Xenopus* oocytes 2–7 days before recording. Oocytes were impaled with electrodes containing 3 M KCl (resistance $2\text{--}3 \text{ M}\Omega$) and held at -80 mV in modified Ringer solution containing (in mM) NaCl 150 , KCl 2.8 , HEPES 10 , MgCl₂ 2 , BaCl₂ 1 , pH 7.2 (Ba replaced Ca to prevent the activation of the endogenous calcium-dependent chloride current). Compounds were bath-applied at $3\text{--}4 \text{ ml min}^{-1}$ (bath volume 0.4 ml) at room temperature ($19\text{--}21^\circ\text{C}$).

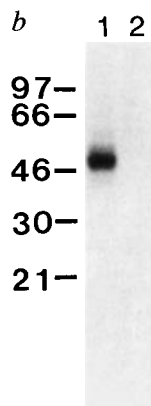


FIG. 1 **a**, Primary sequence of P2X₃ aligned with 2 other known members of the family isolated from PC12 cells and rat vas deferens^{11,12}. Dashes represent sequence identity, dots represent gaps. The putative transmembrane regions are underlined, and the H5-related regions also present in potassium channels are italicized and underlined. Note the conserved cysteine residues (bold) marked with asterisks. **b**, *In vitro* transcription/translation (Promega-TNT system) generates a protein of M_r 45K as predicted by the open reading frame. Track 1, P2X clone; track 2, control (no plasmid DNA). **METHODS.** DRGs from all spinal levels of neonatal Sprague-Dawley rats were isolated, and RNA extracted by using guanidinium isothiocyanate and

acidic phenol extraction¹³. Poly(A)⁺ RNA was isolated by chromatography on oligo(dT)-cellulose and used to generate a directionally cloned oligo(dT)-primed rat DRG cDNA library. cDNA was generated by reverse transcription with oligo(dT)-*Xho*I primer adaptors, and ligated to *Eco*RI adaptors. The $2\text{--}4\text{-kb}$ fraction of *Eco*RI-*Xho*I cDNA was isolated by gel electrophoresis and ligated into lambda zap II (Stratagene synthesis kit). The size-fractionated library was screened by hybridization with a [³²P]dATP random-primed (Gibco Rad-prime kit) P2X-like *Bam*HI probe (positions 1,607–2,524) of the cDNA¹³. The filters were given a final wash in $0.2 \times \text{SSC}$, 0.5% SDS at 65°C . [³⁵S]methionine-labelled proteins were separated on 10% SDS-PAGE (Laemmli) in the presence of reducing agents, autoradiographed with Kodak X-omat film, and relative molecular masses determined with reference to Rainbow molecular mass markers (Amersham).

Hydropathicity plots predict two transmembrane domains, similar to those of the other channels of this class^{11,12}. The amino-acid sequence is similar around these domains but relatively divergent elsewhere (47% identity to the PC12 cell clone P2X₂ and 43% identity to the vas deferens P2X₁). P2X₃ showed some homology with one of the predicted nucleotide-binding sites characterized in PC12 cells between the H5 and M2 domains¹². Some putative extracellular regions (residues 168–179 and 277–287) were conserved between all three proteins. Interestingly, the position of cysteine residues is conserved between different P2X receptors, suggesting conservation of tertiary structure. In contrast, amiloride-sensitive sodium channel subunits and *Caenorhabditis elegans* degenerins have a distinct pattern of expression of cysteine residues, in the predicted extracellular domain¹⁴.

We examined the functional characteristics of this protein by *in vitro* transcription followed by translation of the cRNA in *Xenopus* oocytes¹⁵. Two-electrode voltage-clamp experiments showed that the clone encoded a functional ATP-gated channel, which may be multimeric, as judged by a Hill slope of 1.4 ± 0.1 ($n=15$) at low agonist concentrations. Application of ATP resulted in an inward current (17 ± 5 nA at 100 nM, and -80 mV; $n=15$) with a reversal potential of -7.9 mV ($+1.3$; $n=3$) and marked inward rectification (Fig. 2), consistent with an inward cation flux. As the agonist concentration was increased, the current had a faster rise time and showed a more pronounced and faster sag in response to sustained agonist application. The apparent concentration for half-maximal response (EC_{50}) for ATP was $1.2 \mu\text{M}$ ($n=5$), but the precision of this estimate is limited by the profound desensitization observed. This recovered slowly, and to obtain a consistent response to

moderate ATP concentrations a minimum interval of 15 minutes between applications was adopted. The channel was activated by a range of ATP-related compounds (Fig. 2e–g). The rank order of potency at low concentrations was 2-methylthio-ATP $\gg$ ATP $>$ α,β -methylene-ATP $>$ ATP- γ S $>$ 2'-deoxy-ATP $>$ CTP $\approx$ ADP $\gg$ UTP $\approx$ β,γ -methylene-ATP $>$ GTP. A number of other compounds (100 μM acetylcholine, 50 μM serotonin, 100 μM AMP and 4 μM capsaicin) did not activate the channel ($n=3$).

Suramin, a potent analgesic agent that is commonly used for its anti-trypanosomal activity¹⁶, reversibly reduced responses to 50 nM ATP by $78.4 \pm 5.5\%$ at 1 μM ($n=4$). Thus the properties of oocyte-expressed P2X₃ channels were very similar to those of rat sensory neurons, namely fast and pronounced desensitization with a slow recovery, inward rectification and distinctive agonist pharmacology, particularly with respect to the relative potencies of 2-methylthio-ATP, ATP, α,β -methylene-ATP and β,γ -methylene-ATP^{2,4,5,7,8,17,18}, suggesting that the expression of this channel may account for the P2X receptor activity defined in sensory neurons.

Using northern blots, we found that the P2X₃ mRNA is undetectable in RNA isolated from tissues other than neonatal and adult rat DRG. When neonatal rats were treated with capsaicin and total adult DRG RNA subsequently examined by northern blotting, the signal was substantially reduced, suggesting that the P2X₃ transcript is selectively expressed by capsaicin-sensitive neurons that are predominantly nociceptors (Fig. 3). Only the pituitary showed a detectable P2X₃ signal after long exposure of northern blots. P2X₁ was also present at very low levels in DRG. P2X₂ was present at levels of about 40-fold less than the P2X₃ transcript in DRG RNA,

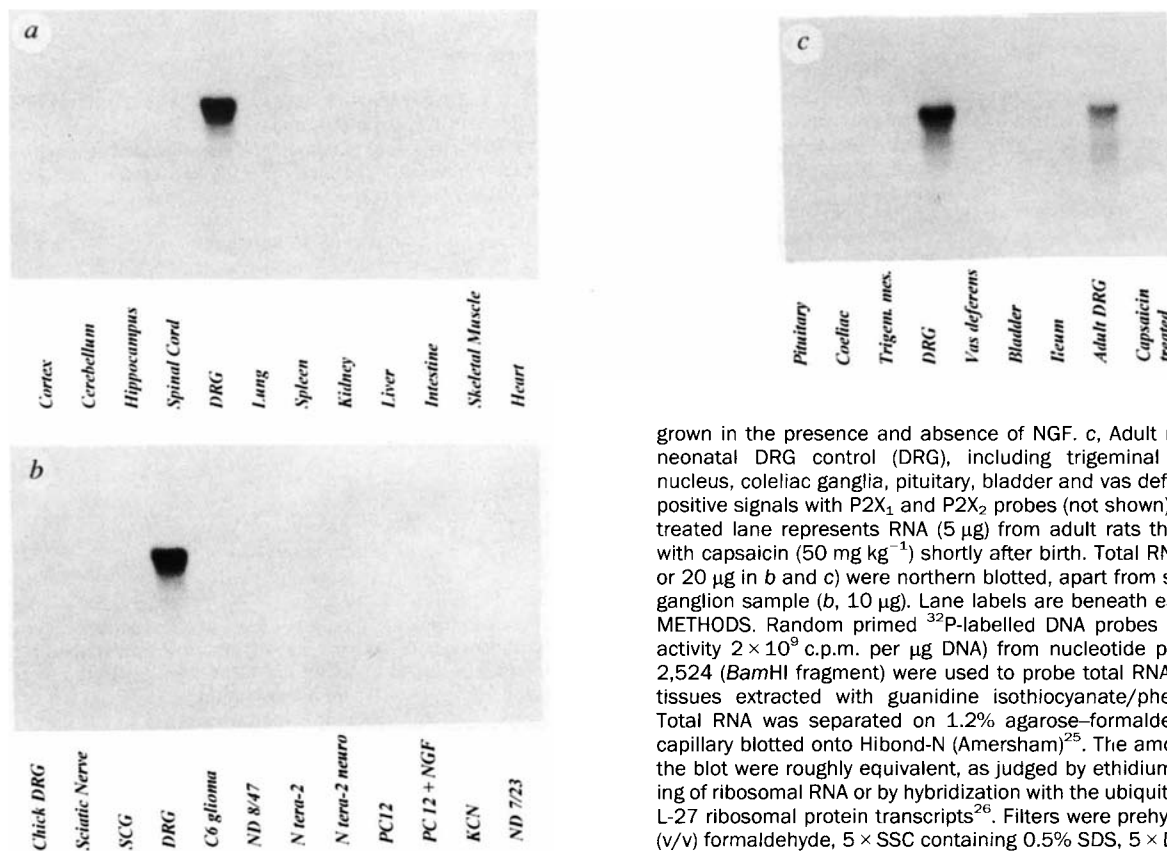


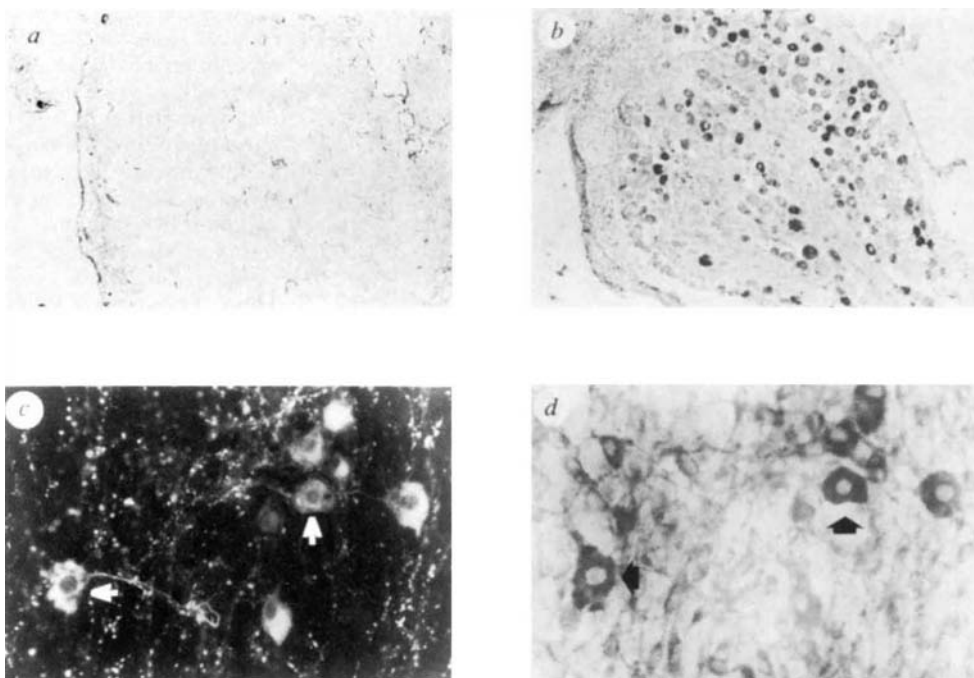
FIG. 3 Northern blot of P2X₃ distribution in neonatal and adult rat tissues and cell lines. a, Central nervous system and non-neuronal tissues from neonatal rats. b, Cell lines tested were C6 glioma, human embryonal carcinoma line N-tera-2 and N-tera-2 neuro²³, rat sensory neuron-derived lines ND7 and ND8 (ref. 24), and human neuroblastoma SMS-KCN. All cells tested gave negative results, including PC12 cells

grown in the presence and absence of NGF. c, Adult rat tissues, with neonatal DRG control (DRG), including trigeminal mesencephalic nucleus, coeliac ganglia, pituitary, bladder and vas deferens that show positive signals with P2X₁ and P2X₂ probes (not shown). The capsaicin-treated lane represents RNA (5 μg) from adult rats that were treated with capsaicin (50 mg kg⁻¹) shortly after birth. Total RNAs (10 μg) in a, or 20 μg in b and c) were northern blotted, apart from superior cervical ganglion sample (b, 10 μg). Lane labels are beneath each panel.

METHODS. Random primed ³²P-labelled DNA probes (50 ng), specific activity 2×10^9 c.p.m. per μg DNA) from nucleotide positions 1,607–2,524 (BamHI fragment) were used to probe total RNA extracted from tissues extracted with guanidine isothiocyanate/phenol/chloroform. Total RNA was separated on 1.2% agarose-formaldehyde gels, and capillary blotted onto Hibond-N (Amersham)²⁵. The amounts of RNA on the blot were roughly equivalent, as judged by ethidium bromide staining of ribosomal RNA or by hybridization with the ubiquitously expressed L-27 ribosomal protein transcripts²⁶. Filters were prehybridized in 50% (v/v) formaldehyde, 5 $\times$ SSC containing 0.5% SDS, 5 $\times$ Denhardt's solution, 100 $\mu\text{g ml}^{-1}$ boiled salmon-sperm DNA, 10 $\mu\text{g ml}^{-1}$ poly(U) and 10 $\mu\text{g ml}^{-1}$ poly(C) at 45 $^{\circ}\text{C}$ for 6 h. After 36 h of hybridization in the same conditions using 10^7 c.p.m. per ml hybridization probe, the filters were washed briefly in 2 $\times$ SSC at room temperature, then twice with 2 $\times$ SSC with 0.5% SDS at 68 $^{\circ}\text{C}$ for 15 min, followed by a 20-min wash in 0.5% SDS, 0.2 $\times$ SSC at 68 $^{\circ}\text{C}$. The filters were autoradiographed overnight on Kodak X-omat film on the blots shown here.

FIG. 4 *In situ* hybridization carried out with digoxigenin-labelled cRNA transcripts from unique P2X₃ sequence. *a*, Control using sense probe. *b*, Bright-field view $\times 25$ of 7- μ m section of adult rat dorsal root ganglia. Note the intensely positive small-diameter cell bodies. Magnification, $\times 25$. *c*, Immunofluorescence staining of peripherin²⁷ detected with a polyclonal rabbit antiserum. Note the precise coincidence between peripherin positive cells (predominantly C-fibres) and P2X₃-transcript-positive cells, confirming that the *in situ* hybridization stain does not block immunofluorescent antigen detection. *d*, The same section as *c* showing a phase-contrast micrograph of primary cultures of neonatal rat dorsal root ganglia grown for 5 days in the presence of NGF as described²⁸ but without anti-mitotic agents, showing darkly stained neuronal cell bodies after *in situ* hybridization. Magnification, $\times 100$.

METHODS. A P2X₃ *Bam*HI fragment between positions 2,318 and 2,524 was subcloned into pGem4z, and digoxigenin-UTP-labelled sense or antisense cRNA generated. Sample preparation, hybridization, and visualization of *in situ* hybridization with alkaline-phosphatase-conjugated anti-DIG antibodies was carried out



as described²⁹. Immunocytochemical staining of cultures and sections was done after *in situ* hybridization with RT97 or rabbit anti-peripherin²⁷.

and was also found in trigeminal mesencephalic nucleus and sympathetic neurons of the superior cervical ganglion. Thus P2X₃ is the only P2X receptor exclusively expressed by a subset of crest-derived sensory neurons. The large excess of P2X₃ mRNA over P2X₂ messenger RNA in DRG cells, if mirrored at the level of protein, suggests that P2X₃ must represent the major component of DRG ATP-gated channels.

Electrophysiological studies have suggested that between 40 and 96% of DRG sensory neurons in culture respond to ATP with elevated intracellular free calcium concentrations, or depolarizing responses^{6,7,9,17,18}. We examined the pattern of expression of the P2X₃ transcript in cells of rat DRG, by double-labelling *in situ* hybridization/immunocytochemistry, using the monoclonal antibody RT97 that stains large-diameter sensory neurons¹⁹. These studies showed that P2X₃ transcript was present in RT97-negative sensory neurons, that correspond to C-fibre afferents, of which 50–90% are polymodal nociceptors. We then used primary cultures to count the number of DRG neurons expressing the transcript, and correlate this with the expression of peripherin, a cytoskeletal protein associated with small-diameter sensory neurons. The transcript is expressed selectively in a subset of DRG neurons (>75% of peripherin-positive cells, and <10% of RT97-positive cells), as well as small-diameter cell bodies of the adult trigeminal ganglion. The total number of neurons in adult rat L4 dorsal root ganglia that expressed high levels of P2X₃ transcript was $25 \pm 5\%$, and a further $31 \pm 6\%$ of neurons expressed above background signals (Fig. 4). This number is less than the figure of up to 90% of neurons that have been observed to respond to ATP electrophysiologically in tissue culture^{6,17}. These data demonstrate that P2X₃ is expressed by small-diameter sensory neurons, most of which are nociceptors, consistent with the capsaicin-ablation experiment, and suggest that some of the responses to ATP measured in large-diameter sensory neurons *in vitro* may be the result of activation of other pathways.

The properties of the P2X₃ receptor described here thus correspond closely with those described for the channel found on

sensory neurons^{9,18}. Activation of this channel provides a plausible molecular explanation for the pain-producing actions of ATP¹⁰, and the analgesic actions of suramin. In addition, the possibility that P2X₃ may contribute to heteromultimeric channels involved in responses to other ligands, in mechanoreception or in programmed cell death^{14,20,21} will now be testable. □

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Coexpression of P2X₂ and P2X₃ receptor subunits can account for ATP-gated currents in sensory neurons

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Cation-selective P2X receptor channels were first described in sensory neurons¹⁻⁴ where they are important for primary afferent neurotransmission and nociception^{5,6}. Here we report the cloning of a complementary DNA (P2X₃) from rat dorsal root ganglia that had properties dissimilar to those of sensory neurons. We also found RNA for (P2X₁) (ref. 7), (P2X₂) (ref. 8) and P2X₄ (ref. 9) in sensory neurons; channels expressed from individual cDNAs did not reproduce those of sensory ganglia. Coexpression of P2X₃ with P2X₂, but not other combinations, yielded ATP-activated currents that closely resembled those in sensory neurons. These properties could not be accounted for by addition of the two sets of channels, indicating that a new channel had formed by subunit heteropolymerization. Although in some tissues responses to ATP can be accounted for by homomeric channels^{1,7-10}, our results indicate that ATP-gated channels of sensory neurons may form by a specific heteropolymerization of P2X receptor subunits.

We obtained a single cDNA (P2X₃ receptor) from a rat dorsal root ganglion (DRG) library which encoded a 397-amino-acid protein having 41–46% identity with other P2X receptors (Fig. 1a). Expression of the P2X₃ receptor in HEK293 cells showed that its properties differed from those of sensory neurons in three important respects. First, ATP and $\alpha\beta$ -meATP ($\geq 1 \mu\text{M}$) evoked rapidly desensitizing currents (Fig. 2a), whereas these agonists evoke non-desensitizing currents in cultured rat nodose (Fig. 2b) and DRG neurons^{11,12}. Second, the P2X₃ current activated considerably faster than observed with the same concentrations of agonist in DRG¹¹ or nodose ganglion cells¹³ (Fig. 2a, b). Third, the kinetics of PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid) inhibition was significantly faster than in nodose neurons¹⁴. That is, currents induced by ATP (30 μM) were maximally inhibited by 100 μM suramin and 30 μM PPADS; half-maximal inhibition occurred at $3 \pm 1.2 \mu\text{M}$ ($n=6$) and $1.5 \pm 0.6 \mu\text{M}$ ($n=7$), respectively. The inhibition by PPADS developed rapidly (<30 s) and washed out within 2 min, unlike the very slow kinetics of block observed with P2X₁ and P2X₂ receptors and nodose ganglion neurons^{13,14}.

Concentration-response curves for ATP, $\alpha\beta$ -meATP, 2-methyl-thio-ATP, ATP- γ -S and ADP to activate the P2X₃ receptor were similar to those for activating the endogenous nodose ganglion receptors^{12,13} (Figs 2c and 3c). ATP currents at the P2X₃ receptor reversed at -1.6 ± 0.4 mV ($n=11$) in standard recording solutions (Fig. 2a) and at 12.4 ± 2.1 mV ($n=7$) when all external cations were replaced with CaCl_2 , demonstrating that this receptor is also a non-selective cationic channel with a high calcium permeability¹ ($P_{\text{Ca}^{2+}}/P_{\text{Na}^+}$ ratio ≈ 4).

Because the properties of the P2X₃ receptor could not account for those of sensory neurons, we examined whether any of the

FIG. 1 a, Predicted amino acid sequence of P2X₃ (3) receptor isolated from sensory ganglia aligned with P2X₂ (1) and P2X₂ (2) receptors; overlines indicate hydrophobic, putative transmembrane domains. The identity and similarity to the other receptors is dispersed throughout the sequences but the hydrophobicity plots were almost superimposable (not shown). b–d, *In situ* hybridization in the adult rat nodose ganglia with dioxigenin-labelled antisense cRNA probes for P2X₂ (b), P2X₃ (c) and P2X₄ (d). Similar results were obtained when antisense cRNA probe for the P2X₁ receptor was used (not shown). Serial sections are shown; in some instances the same cell with RNA staining can be identified in all three sections, providing direct indication that RNA for all three receptors are present. Neuronal cells were identified and counted using DAPI or neutral red stain for nuclei. Hybridization for each P2X mRNA was detected in 210–220 of 223 cells classified as neurons; counts were made from serial sections. Scale bars, 50 μm .

METHODS. A 440-bp fragment of P2X₃ cDNA was initially isolated from superior cervical ganglia cDNA by polymerase chain reaction (PCR). This fragment was used as a hybridization probe to screen a λ ZAP cDNA library, prepared from the mRNA of rat dorsal root ganglia. A 1753-bp cDNA was subcloned from lambda into pcDNA3 (Invitrogen, San Diego) and sequenced (accession number X91167) by fluorescent DNA sequencing (Applied Biosystems). For *in situ* hybridization, 6- μm sections were fixed in 4% paraformaldehyde and hybridized at high stringency

a

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1: MARRLQDELSAFFFEYDTPRMVLVRNKKVGVIFRLIQLVVLVVGWVVEYKGYQTSS-GLISSVSVKLGAVTQLQG 079
3: M-----NCISDFFTKYETTKSVVVKSWTIGIINRAVOLLITSFVGVVFLHERAYVRDTAIESVVTXKVGFGRYAN-- 078
2: MVRRLARGCWSAFAWDEETPKVIVVRNRRILGFVHRMVQLLLILFVWVVEIVQSYQDSETDPESSITTKVKGITMSD-- 072

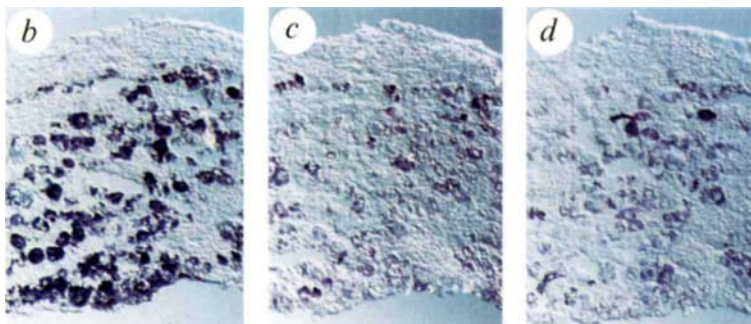
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2: ---KVWDVEEYKPEPEGGSVVSITRIEYTPSOTLSTCPSSMRVHSSTCHSDDDCIAGQLDMQGNIGRTGHCVPYTHGDS 155

1: KTCETIFGNCQFVEVDDKIPSPALLREANFELFIRKNSISFPERFVNRNRLVEEVNGTYMKKCLYHKIQHLEFVFNLYGVV 236
3: RTCEIQGMCPTFVD-TVMFIMM-EAENETIFKNSIRFELFNFKEGNLPLNTDKDIRCRHFEKAFKPCILRVGDV 228
2: KTCGVSAWCEVE-DGTSDNHFLGKMAPNETILKNSIHYEKKFKSKOMIASQ-KSDTYLHCTEDQDSDYQPPFRLGFI 233

1: RESGQDFRSLAEKGGVVGITIDMKCOLDWVHRHCKFIYQEHGYG---EKNLSPGCFNFRARHFVQNG-TNRRHLEFVFG 312
3: KFACQDFAKLARTGGVLGKIGWVCDLKWQDCIEKYSTRLDGVSEKSSVPGYNFRFAKYKKNENGSEYRTLLKAFG 308
2: EKAGENETELAHKGGVIGVIINNNCDLSESECNKYSERRRDPKY--DPAESGYNFRFAKYKINGTTTTLIKAYG 311

1: IHFDILVDGKAGKEDIIPMTTIGSGIGIFGVATVLCDLLLHILPERRHYKOKKKFYAEDMGPGEGEHDPVATSSSTLGL 392
3: IRFDVLVYGNAGKFNIIPTIISVAAFTSVGVGTVCDDIILNFKGADHYKARKFEVETETTLKGASTNPVFPASDAQ 388
2: IRIDVIVHGOAGKFSLEPTIINLATLTSIGVGSFLCDWILLTFMKNKLYSHKKFDKVRTPKHPSSRWVPTLALVLGQI 391

1: QENMRTS 399
3: VEKQSTDGAYSIGH 397
2: PPPSHYSQDQPPSPSPSGEGPTLGEAGELPLAVQSPRPCISALTEQVVDTLGQHMGQRPPVPEPSQDSTSTDPKGLAQL472
  
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(50% formamide at 72 °C) with 50 ng ml⁻¹ of digoxigenin-UTP-labelled full-length cRNA probe. Hybridization was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase reacting with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer). Results are shown for 18-hr phosphatase reactions. Sections were photographed with Nomarski optics. Sense strand controls were uniformly negative.

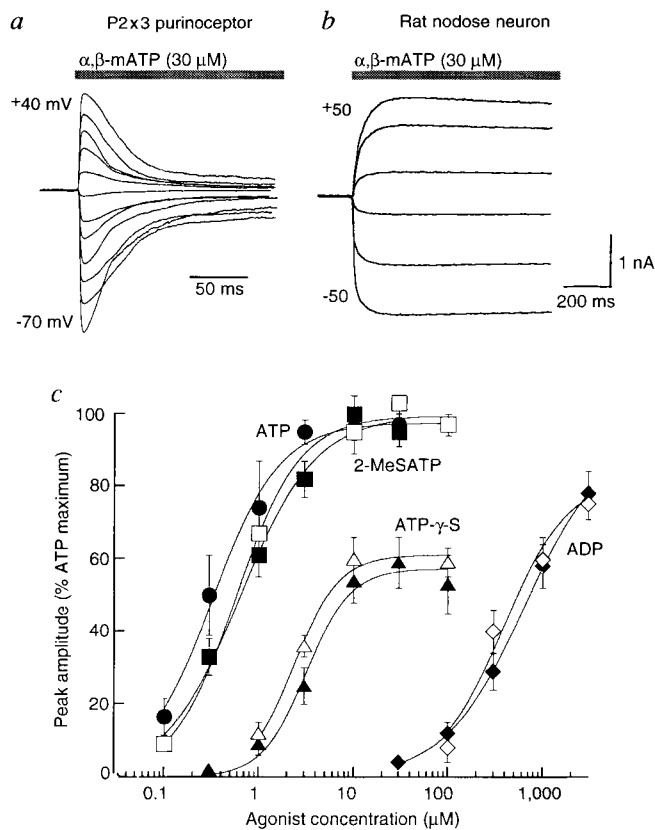


FIG. 2 Functional properties of heterologously expressed P2X₃ receptor compared with native receptor in sensory neurons. *a*, Currents recorded from HEK293 cells transfected with P2X₃ cDNA in response to fast-flow application of $\alpha\beta$ -meATP (30 μ M, applied for duration of bars above traces) at 10 mV interval holding potentials from -70 to +40 mV; note the rapid onset and decay during first 50 ms of agonist application. For P2X₃, the decay of the response during agonist application at this concentration was best fit to the sum of two exponentials (τ_{d1} and τ_{d2}). *b*, Similar experiment performed on rat nodose neuron; note the slower onset rate ($\tau_{on} = 24 \pm 2$ ms, $n = 15$) and no desensitization of current. *c*, Concentration-response curves for ATP, 2-MeSATP, ATP- γ -S and ADP in activating P2X₃ receptors heterologously expressed in HEK293 cells (filled symbols as indicated) and nodose neurons (open symbols). **METHODS.** Conventional whole-cell recording and fast-flow U-tube agonist application methods were used. Standard internal pipette solution was K-aspartate or Cs-aspartate 140 mM, NaCl 20 mM, EGTA 10 mM, HEPES 5 mM; standard external solution was NaCl 147 mM, CaCl₂ 2 mM, MgCl₂ 1 mM, KCl 2 mM, HEPES 10 mM and glucose 12 mM. Solutions for experiments in which calcium permeability ratios were determined were as previously described⁷. Lipofectin method was used for transient transfection with 1 μ g ml⁻¹ cDNA per 8×10^4 HEK293 cells; total cDNA concentration per cell count was kept constant so that in coexpression experiments 0.5 μ g per 8×10^4 cells of each cDNA was added. Recordings were obtained 12–48 h after transfection. Recordings from adult rat nodose neurons were obtained 1–5 days after dissociation, as described previously¹³.

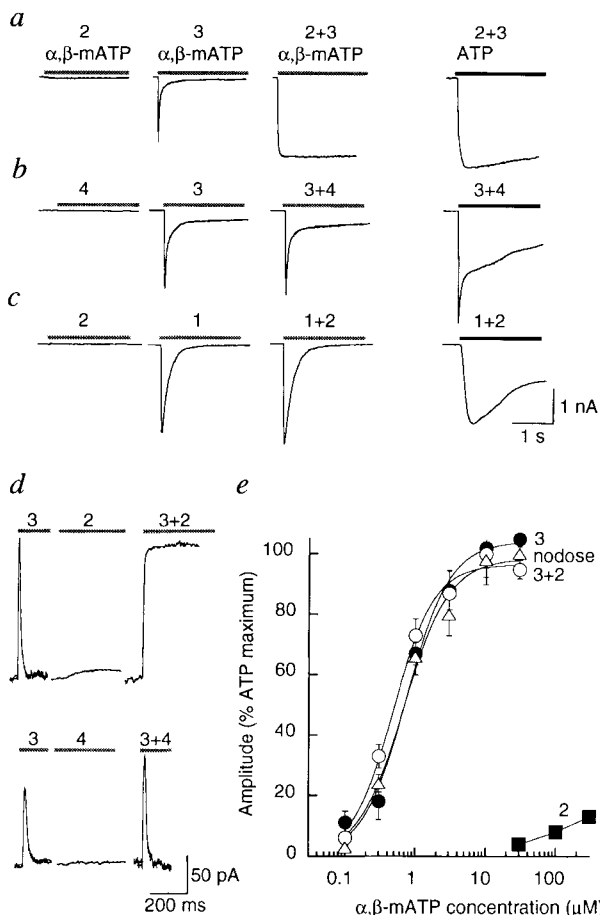
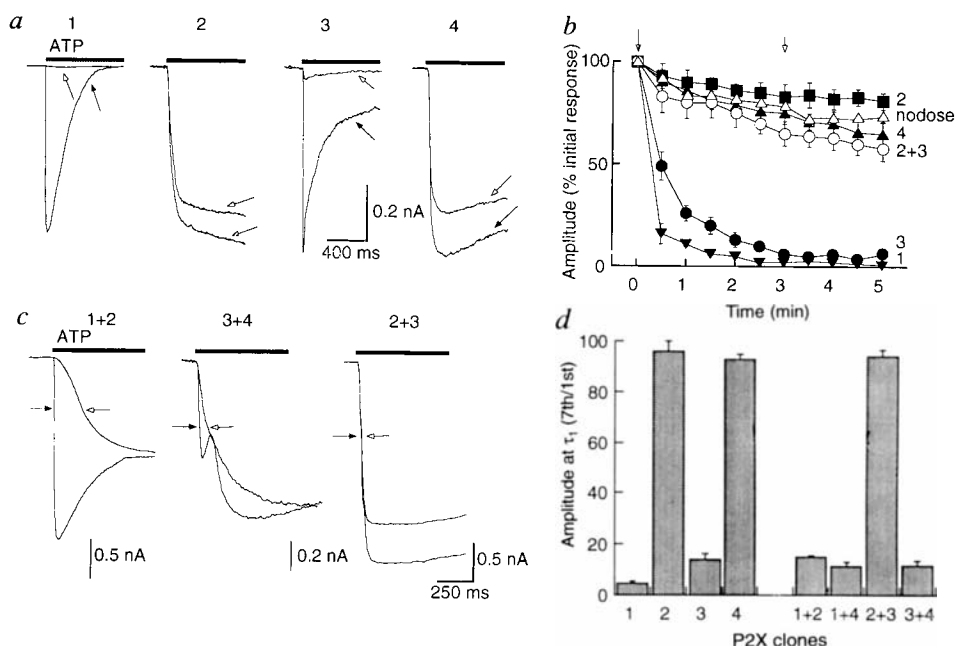


FIG. 3 Coexpression of P2X₃ (3) with P2X₂ (2), but not other combinations, yields a non-desensitizing current which is activated by $\alpha\beta$ -meATP. *a*, Representative recordings obtained from HEK293 cells transfected with P2X₂ alone, with P2X₃ or with P2X₂ and P2X₃. Desensitization of the current occurs in cells expressing P2X₃ alone but not in cells expressing P2X₂ and P2X₃ together. Time constants for activation (τ_{on}) and desensitization (τ_d) for P2X₃ were $\tau_{on} 1.9 \pm 1.0$ ms, $\tau_{d1} 86 \pm 11$ ms and $\tau_{d2} 1.3 \pm 0.9$ s ($n = 12$), and for P2X₂ and P2X₃ together they were $\tau_{on} 27 \pm 3$ ms and $\tau_d 14 \pm 4$ s ($n = 36$), respectively. *b*, *c*, Similar set of experiments in which P2X₄ was cotransfected with P2X₃ (*b*) or P2X₁ (*c*) with P2X₂. Cells expressing both P2X₃ and P2X₄ had τ_{on} of 2 ± 0.8 ms, $\tau_{d1} 93 \pm 15$ ms and $\tau_{d2} 1.1 \pm 0.9$ s ($n = 14$); cells expressing P2X₁ showed $\tau_{on} 5.2 \pm 0.9$ ms, $\tau_d 320 \pm 39$ ms ($n = 24$), whereas cells cotransfected with P2X₁ and P2X₂ showed $\tau_{on} 4.8 \pm 1.1$ ms, $\tau_d 310 \pm 39$ ms ($n = 21$), and P2X₁ and P2X₄, $\tau_{on} 6.0 \pm 2.1$ ms, $\tau_d 400 \pm 85$ ms ($n = 16$). *d*, Traces on the right show recording from same cell in response to ATP; we used these differences in kinetics of responses to $\alpha\beta$ -meATP and ATP as evidence that both receptors were functionally expressed in the same cell (see Fig. 4 legend). Holding potential was -70 mV. *e*, Outward currents evoked by $\alpha\beta$ -meATP in the absence of external calcium and magnesium; holding potential was 50 mV. *e*, Concentration-response curves for $\alpha\beta$ -meATP at P2X₂, P2X₃, P2X₂ and P2X₃ together receptors and in cultured rat nodose neurons. Each point is mean $\pm$ s.e.m. of 6–13 cells, except for P2X₂ in which case $n = 3$.

FIG. 4 Time course of activation of ATP currents and rundown of heterologously expressed P2X receptors. **a**, Each set of traces shows superimposed currents recorded in response to the first (filled arrow) and seventh (open arrow) application of ATP (30 μ M) at 30-s intervals; duration of application is 1 s. **b**, Run-down of current during repeated ATP application for clones P2X₁, P2X₂, P2X₃ and P2X₄ alone, for the P2X₃ and P2X₂ combination, and for nodose neurons as indicated; each point is mean $\pm$ s.e.m. of 4–8 experiments except for P2X₃ and P2X₂, where $n=24$. **c**, Similar experiment to **a** but in HEK cells cotransfected with combinations of P2X receptors as indicated. Note the pronounced change in rise time of responses recorded in cells cotransfected with P2X₁ and P2X₂ together, or P2X₃ and P2X₄ together, but not in cell expressing P2X₂ and P2X₃ combination. **d**, Summary of results obtained from all experiments as illustrated in **a** and **c**. For clones expressed alone, time constant of onset (τ_{on}) was measured in each cell for the current evoked by the initial application and this time was used to measure the amplitude of the seventh response. For combinations, the mean value of τ_{on} obtained for the 'fast-rising' clones (P2X₁ and P2X₃) expressed alone was used as the point at which amplitudes were measured for the first and seventh response.

METHODS. It is possible that both sets of channels were not expressed in cells cotransfected with cDNAs. Specifically, where $\alpha\beta$ -meATP evoked a fast-desensitizing response (P2X₁ and P2X₃) and a similar response was seen after cotransfection with P2X₂ or P2X₄ it was necessary to show that P2X₂ or P2X₄ also were being expressed. This was done in two ways. First, in all cells the response to $\alpha\beta$ -meTP (30 μ M) was compared to the response to ATP (30 μ M). Coexpression was assumed when the response to ATP desensitized more slowly than the response to



$\alpha\beta$ -meATP because with P2X₁ and P2X₃ alone responses to ATP and $\alpha\beta$ -meATP desensitize at the same rate. Simulations indicated that this effect of P2X₂ or P2X₄ would be detectable if they provide more than 10% of the total current. Second, in all cells ATP was applied repeatedly until (seventh application) the response at the P2X₁ or P2X₃ receptor had completely rundown. With clones expressed alone, this rundown was not associated with any change in τ_{on} ; therefore, a slowing of the time constant of activation during the rundown was taken as evidence for the presence of P2X₂ or P2X₄. Simulations indicated that this method would detect the presence of P2X₂ or P2X₃ if they contributed more than 30% of the total current; we rejected 7 of 73 cells as providing no evidence that both receptor subunits had expressed.

other cloned P2X receptors were expressed by these cells. *In situ* hybridization of rat nodose and DRG neurons showed messenger RNA for all four receptors in 90–95% of these neurons (Fig. 1b–d). We therefore investigated whether coexpression of any two of the four P2X subtypes caused an ATP-gated current which exhibited minimal desensitization but was fully activated by $\alpha\beta$ -meATP.

Fast-rising, desensitizing currents are evoked by 30 μ M $\alpha\beta$ -meATP at P2X₁ and P2X₃, but not P2X₂ and P2X₄, receptors^{1,7–10,14}. We therefore tested its action on cells transfected with P2X₁ or P2X₃ together with P2X₂ or P2X₄. Coexpression of P2X₃ with P2X₂ led to $\alpha\beta$ -meATP-induced currents that activated more slowly than those seen with expression of P2X₃ alone, and the current desensitized negligibly during application (lasting 2 s) of $\alpha\beta$ -meATP (Fig. 3a). Because P2X₂ does not respond to $\alpha\beta$ -meATP when expressed alone, the difference in properties of P2X₂ and P2X₃ together, as opposed to P2X₃ alone, indicates the formation of channels of a new phenotype. This phenotype ($\alpha\beta$ -ATP-sensitive, non-desensitizing) is very similar to that seen in sensory neurons. In contrast to P2X₂ and P2X₃ together, the responses to $\alpha\beta$ -meATP were the same as P2X₃ when P2X₃ and P2X₄ were coexpressed (Fig. 3b). In similar experiments, no evidence was found for the rise time or desensitization of P2X₁ being altered by coexpression with P2X₂ or P2X₄ (Fig. 3c). For all combinations, similar results were obtained for outward currents recorded at 50 mV (Fig. 3d), as well as for currents recorded in the absence of external calcium or magnesium ($n=3$ for each combination; Fig. 3d) and when intracellular BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) (10 mM) was used ($n=2$ for each combination). Peak currents and concentration–response curves to $\alpha\beta$ -meATP were the same in cells transfected with P2X₃ alone, P2X₂ and P2X₃,

together (Fig. 3e), P2X₃ and P2X₄, P2X₁ alone, P2X₂ and P2X₁, and P2X₄ and P2X₁. The $\alpha\beta$ -meATP induced current in cells transfected with P2X₃ and P2X₂ was inhibited by $80 \pm 4\%$ by PPADS (10 μ M, $n=6$), but the inhibition was only partly reversible within 15 min of washing ($52 \pm 11\%$).

Whereas $\alpha\beta$ -meATP activates only P2X₁ and P2X₃, ATP activates all four P2X receptors with ATP concentration–response curves differing by less than tenfold among clones^{7,10,14} (Fig. 2c). However, with repeated applications of ATP at 30-s intervals, the evoked current declines by more than 95% in the case of P2X₁ and P2X₃, but there is little or no such 'rundown' in the response with P2X₂ and P2X₄^{7,10,11,15,16} (Fig. 4a). If channels are expressing as separate populations in cotransfection experiments, the component contributed by P2X₁ or P2X₃ should disappear during repeated applications at 30-s intervals. Because P2X₁ and P2X₃ rise more quickly than P2X₂ and P2X₄, the most sensitive indicator for such a loss of their component would be a significant slowing of the rise time (Fig. 4 legend). When we measured rise times during rundown experiments, with P2X₃ and P2X₂ together there was little or no change in the rising phase of the response from the first to the seventh application, and the peak response showed minimal rundown ($n=21$ of 24 cells; Fig. 4b–d). However, in the combined expression of P2X₄ and P2X₃, P2X₁ and P2X₂, or P2X₁ and P2X₄, an initial fast component to the rise time disappeared during the repeated applications, and τ_{on} (the time constant of onset) measured from the current evoked by the seventh application was not significantly different from the averaged τ_{on} measured in cells transfected with P2X₄ or P2X₂ alone ($n=12$ for each combination). These results indicate that subunits P2X₁ and P2X₂, P2X₁ and P2X₄, or P2X₃ and P2X₄, behave as independent sets of channels, whereas P2X₃ and P2X₂ together shows a rise time and

rundown phenotype similar to P2X₂ alone. Thus, in the P2X₂ and P2X₃ heteropolymer, the P2X₂ subunit is dominant with respect to rise time, desensitization and rundown of the response to ATP as well as PPADS action, but the P2X₃ subunit confers to the channel its agonist sensitivity.

The heteropolymerization of P2X₃ and P2X₂ subunits provides a channel that is similar in its properties to those observed in rat nodose and DRG cells^{1,4,11,13} (Figs 2c, 3a, e and 4b). We cannot exclude a contribution to the native channels from additional P2X subunits not yet cloned, but the most parsimonious explanation is that sensory neurons express channels which are composed minimally of P2X₂ and P2X₃ subunits. Although RNAs for all four receptors are found in almost all ganglion cells, it is possible that some are transported preferentially away from the cell soma; P2X₁ receptors have been located by immunohistochemistry in primary afferent terminals within the spinal cord¹⁷.

These results provide the first evidence that P2X receptors are multimers, in which subunits can assemble as either homo- or heteropolymers. This has clear parallels to nicotinic¹⁸ and glutamate¹⁹ receptor channels, and voltage-gated potassium channels²⁰. In the case of voltage-gated potassium channels, heteropolymerization occurs only among subunits belonging to the major phylogenetic subfamilies (*Shaker*, *Shab*, *Shaw* and *Shal*)²⁰ a similar situation could become evident for P2X receptors as the sequences of more family members become available.

The precise subunit composition of the sensory neuron P2X receptor presumably provides a phenotype that is attuned to the role of ATP in the generation and/or transmission of primary afferent information. □

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***Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor**

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THE Gram-positive bacterium *Streptococcus pneumoniae* is a major cause of pneumonia, sepsis and meningitis¹. Although the invasive disease is severe, some 40% of individuals harbour the pneumococcus in the nasopharynx asymptotically². Here we investigate the molecular elements of the encounter between host and pathogen that distinguish these different outcomes. We show that inflammatory activation of human cells shifts the targeting of the pneumococcus to a new receptor, that for the G-protein-coupled platelet-activating factor (PAF). Only virulent pneumococci engage the PAF receptor. Attachment of the bacterial phosphorylcholine to the PAF receptor enhanced adherence, which was coupled to invasion of endothelial, epithelial and PAF-receptor-transfected cells. This progression could be arrested *in vitro* and *in vivo* by PAF-receptor-specific antagonists, suggesting a possible approach to therapy.

Pneumococci adhere to but fail to enter resting human endothelial cells but can be visualized inside an intracellular compart-

ment upon inflammatory activation of the cells³. To determine whether activation affords pneumococci a specific route of translocation into endothelial cells, we allowed pneumococci to adhere to cytokine-activated endothelial cells and the number of internalized bacteria was measured by protection from killing by exogenous gentamicin (Fig. 1). Only 0.1% of the adherent bacteria were recovered from lysates of resting cells. Activation of endothelial cells with thrombin or tumour-necrosis factor (TNF)- α caused a 20–40-fold increase in bacterial entry into cells (2.3 and 3.7% of adherent bacteria, respectively). Activation of endothelial cells results in expression of novel surface determinants important for leukocyte trafficking, including PAF and PAF receptor^{4–11}. The PAF receptor is known to be rapidly internalized after interaction with ligand¹², and PAF¹³ and the pneumococcal cell wall¹⁴ share phosphorylcholine as a determinant of proinflammatory activity. Involvement of PAF receptor in the uptake of pneumococci into activated cells was suggested by the ability of a specific PAF-receptor antagonist strongly to deter the internalization of pneumococci into activated endothelia (Fig. 1).

Surface determinants of resting eukaryotic cells containing *N*-acetylglucosamine β -1-4-galactose or *N*-acetylglucosamine β -1-3-galactose can tether pneumococci (Table 1: asialo-GM-2 and globoside), but resting cells do not support translocation^{3,15–17}. As has been shown for leukocyte trafficking⁶, both the quantity and the fate of pneumococci adherent to human cells was affected by activation of the target cell (Fig. 1). An 81% increase in adherence occurred within 10 min of addition of thrombin, decaying back to baseline by 60 min. Increases of 190 and 91% were sustained over hours after TNF or interleukin-1 (IL-1) treatment, respectively. Lung cells were activated for adherence only with IL-1 and to a lesser degree (35%) than endothelial cells (data not shown). Adherence of several encapsulated strains was indistinguishable. Adherence to activated cells was unchanged ($99 \pm 3\%$) by the presence of 50% fresh human serum.

The mechanism of pneumococcal attachment differed between activated and resting cells (Table 1), implicating the PAF recep-

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TABLE 1 Factors inhibiting pneumococcal adherence to activated cells

		Number of pneumococci adherent to 100 host cells (% of control)					
		Active at PAF receptor		Active at pneumococcal surface			Glycoconjugates
Treatment	Control	PAF†	L659,989†	Cell wall†	Ethanolamine‡	TEPC-15§	a-GM2 + globo§
Endothelial cells							
Resting	239 ± 12	248 ± 14 (103%)	237 ± 11 (99%)	120 ± 14 (50%)**	242 ± 13 (101%)	239 ± 15 (100%)	40 ± 8 (17%)**
TNF-α	415 ± 25	244 ± 19 (58%)**	230 ± 15 (55%)**	301 ± 20 (72%)**	121 ± 11 (29%)**	108 ± 18 (26%)**	144 ± 9 (36%)
IL-1α	368 ± 18	254 ± 11 (69%)**	250 ± 10 (68%)**	278 ± 18 (76%)**	130 ± 9 (35%)**	114 ± 12 (31%)**	133 ± 11 (36%)
Thrombin	382 ± 12	207 ± 14 (54%)**	225 ± 12 (59%)**	289 ± 11 (76%)**	136 ± 8 (36%)**	110 ± 15 (29%)**	127 ± 9 (33%)
Lung cells							
Resting	273 ± 14	264 ± 18 (96%)	272 ± 08 (99%)	138 ± 19 (50%)**	294 ± 14 (107%)	275 ± 18 (101%)	64 ± 9 (24%)
IL-1α	355 ± 18	287 ± 16 (80%)*	239 ± 15 (67%)**	249 ± 13 (70%)**	135 ± 13 (38%)**	108 ± 18 (26%)**	125 ± 12 (35%)

* A549 type II lung cells were stimulated with IL-1α (at 5 ng ml⁻¹ for 4 h at 37 °C); endothelial cells were stimulated with TNF-α (10 ng ml⁻¹ for 4 h at 37 °C), IL-1α (5 ng ml⁻¹ for 4 h at 37 °C) or thrombin (2 U ml⁻¹ for 10 min at 37 °C). Adherence of fluorescein-labelled R6 (2 × 10⁷ CFU ml⁻¹) was assessed visually following incubation for 30 min at 37 °C with the monolayers. Values are the means ± s.d. of 6 experiments. ** P < 0.05 compared with control.

† Stimulated monolayers were incubated for 30 min at 37 °C with PAF (50 μM), PAF-receptor antagonist L659, 989 (1 μM), pneumococcal cell wall (50 μg ml⁻¹; ref. 28) or albumin buffer.

‡ R6 pneumococci were grown in defined medium using ethanolamine to replace choline as the aminoalcohol¹⁴.

§ R6 was incubated with either albumin buffer or the antiphosphorylcholine antibody TEPC-15 (for 10 min at room temperature in ascites fluid; from M. Potter). Alternatively, pneumococci were incubated for 15 min at 37 °C with 2 mM (final concentration) of the combination of asialo-GM-2 (a-GM2) and globoside (globo), washed and then co-incubated with activated cells.

|| Less active against activated than resting cells.

tor only in activated cells. PAF pretreatment reduced adherence to activated cells to resting cell levels. PAF receptor antagonist abrogated the enhanced adhesion of pneumococci to cytokine-activated cells in a dose-dependent manner (50% inhibition at 5 × 10⁻⁸ M for L659,989 and at 1 × 10⁻⁸ M for WEB 2086, values

consistent with other models^{14,18}). These findings suggest that virulent pneumococci harbour a cognate ligand for the PAF receptor.

Four observations indicated that the pneumococcal ligand for activated cells could be phosphorylcholine in the cell wall

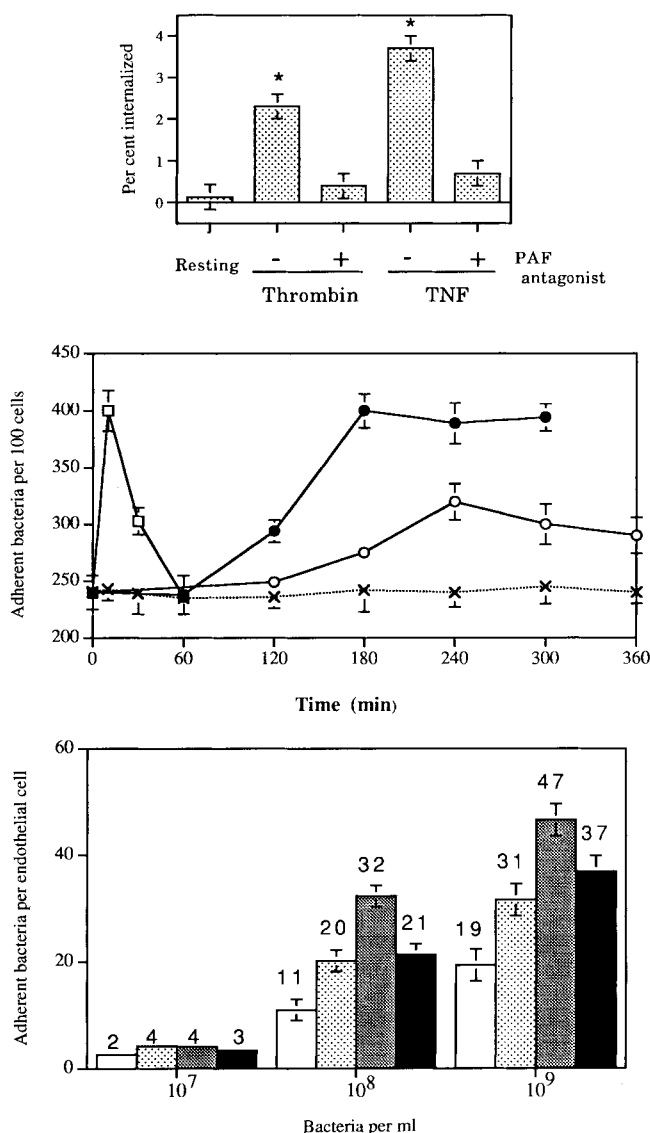


FIG. 1 Potentiation of internalization and adherence of pneumococci by activation of endothelial cells. Top, internalization of pneumococci by resting compared with activated cells. Values are expressed as the per cent of the original adherent population that survived gentamicin and are representative of 4 independent experiments. * P < 0.01 when compared to resting cells and cells treated with PAF antagonist. Middle, effect of activation of endothelial cells on pneumococcal adherence. Endothelial monolayers were stimulated with thrombin (squares), TNF-α (filled circles), IL-1α (open circles) or not stimulated (dotted line). Results are means ± s.d. for duplicate wells in at least 6 independent experiments. Bottom, effect of bacterial concentration on adherence to activated endothelial cells. Monolayers were treated with one of three stimuli as in the middle panel, followed by a varied bacterial inoculum as indicated. White bars, resting cells; stippled bars, thrombin; grey bars, TNF; black bars, IL-1. Values are expressed as the mean ± s.d. of the number of bacteria per individual endothelial cell from 4 experiments.

METHODS. Confluent monolayers of primary human umbilical vein endothelial cells (passage 1; from Clonetics) were incubated with TNF-α (10 ng ml⁻¹ for 3 h at 37 °C; from Boehringer Mannheim⁶) or thrombin (2 U ml⁻¹ for 10 min at 37 °C; from Sigma⁶) or IL-1α (5 ng ml⁻¹ for 4 h at 37 °C; Boehringer Mannheim²⁹). In some experiments, the monolayers were also treated with the PAF-receptor antagonist L659, 989 (at 1 μM for 10 min; Merck Sharp and Dohme Research Labs). *S. pneumoniae* R6 or AII (10⁷ CFU ml⁻¹), grown on trypticase soy agar (from Difco) containing 3% sheep's blood (from Micropure Medical) and labelled with fluorescein isothiocyanate (1 mg ml⁻¹; Sigma)³ were added to the monolayers for 30 min at 37 °C. Non-adherent bacteria were removed by 5 cycles of washing. The number of bacteria adherent to 100 endothelial cells were counted visually. To determine whether internalization had occurred, one of each experimental group of monolayers was lysed immediately to find the number of bacteria adherent to the monolayer. Resting or PAF-receptor antagonist-treated cells supported ~5 × 10⁵ CFU ml⁻¹; stimulated cells supported 11–15 × 10⁵ CFU ml⁻¹. Two other monolayers were treated with 50 μg ml⁻¹ gentamicin (10 × MIC) for 120 min at 37 °C, and then lysed with 0.1% Triton X-100 and plated for colony-forming units to determine the number of bacteria inside cells and therefore protected from antibiotic³⁰. Triton X-100 (up to 1%) was not bacteriocidal.

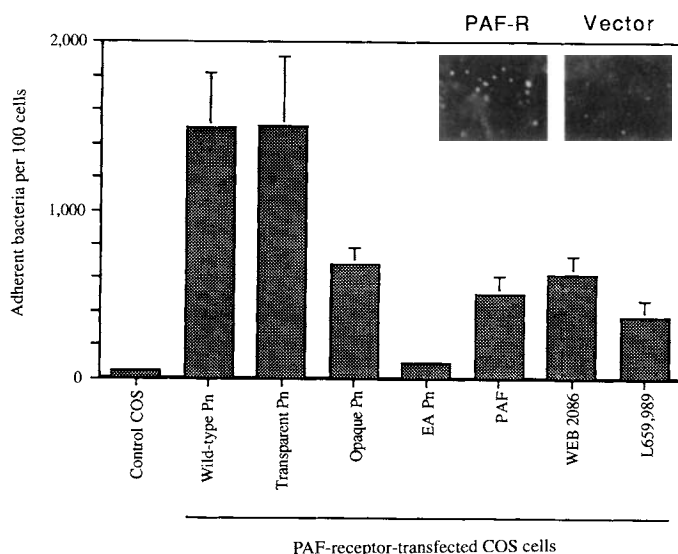


FIG. 2 Adherence of pneumococci to transfected PAF receptor. Adherence of fluorescein-labelled R6 pneumococci (10^8 per ml) to COS cells transfected with the indicated PAF-receptor construct is shown in the inset. Adherence was quantified in the presence of exogenous PAF (10 μ M), or of PAF-receptor antagonists WEB 2086 (10 μ M; Boehringer Mannheim) or L659,989 (10 μ M, Merck, Sharpe and Dohme). Pneumococci (Pn) bearing ethanolamine instead of choline in the cell wall (EA Pn) and isogenic derivatives of wild-type R6 that differed in colonial morphology (transparent and opaque) were also tested.

METHODS. Human myeloid PAF-receptor cDNA was constructed in the expression vector pCDM8 and modified to encode a Flag epitope as described^{12,18}. COS cells were transfected with 2 μ g plasmid in 3 ml Dulbecco's modified Eagle's medium supplemented with 2% NuSerum (Collaborative Research), 1 mM chloroquine (freshly prepared from Sigma) and 400 μ g ml⁻¹ DEAE-dextran (Sigma). Control cells were transfected with vector alone. Cells expressing PAF receptors were determined by immunohistochemical staining of the Flag-PAF receptor with monoclonal antibody^{12,18}.

(Table 1). Pneumococcal binding was reduced to resting levels by replacement of choline by ethanolamine or by incubation with purified cell wall fragments, anti-phosphorylcholine antibody TEPC-15, or 2% choline. Thus, in addition to its ability to trigger the complement¹⁹, cytokine^{3,20} and procoagulant cascades²¹, the cell wall seems to mediate adherence and invasion directly using phosphorylcholine to subvert the PAF receptor.

To determine whether pneumococci adhere to the PAF receptor, COS-7 cells were transfected with human PAF-receptor complementary DNA¹². Transfected cells strongly supported pneumococcal adherence (Fig. 2). Consistent with evidence using native cells, adherence was lost for ethanolamine-grown bacteria and attenuated by exogenous PAF and PAF-receptor antagonists (Fig. 2). Pneumococci phase vary between a virulent, transparent colonial morphology and an avirulent, opaque phenotype²². Avirulent variants adhered poorly to the transfected cells and failed to invade (<0.1% invasion). In contrast, virulent variants adhered strongly and entered 35 times more easily into transfected COS cells (3.4% versus <0.1% for vector transfectant controls); this entry was inhibited to control levels (0.11%) by PAF-receptor antagonist.

Specific PAF receptors, identified in lung, brain, platelets and leukocytes^{4,23,24}, participate in trafficking of leukocytes and generation of bronchospasm and inflammation in asthma²⁵. Endothelial cells show antagonist-reversible biological responses to PAF⁷⁻¹¹, and northern analysis from activated endothelial and epithelial cells confirmed that there was a messenger RNA signal hybridizing with PAF-receptor cDNA (data not shown). To our knowledge, this is the first example of a bacterium invading a eukaryotic cell with the help of G-protein-coupled

receptor^{17,26}. Ligation of the PAF receptor by PAF results in activation of phospholipase C (ref. 27). In accordance with previous results¹⁴, pneumococcal interaction with the PAF receptor failed to stimulate signal transduction, as measured by activation of phosphatidylinositol-specific phospholipase C, and did not interfere with PAF-induced activation of phospholipase C (data not shown). This ability of a ligand to bind receptor in the absence of signal transduction and to enter the cell by apparent subversion of receptor internalization is surprising.

The physiological significance of the pneumococcal-PAF receptor interaction is indicated by the exclusion of avirulent pneumococci from this bioactivity and the ability of PAF-receptor antagonists markedly to attenuate inflammation during experimental pneumococcal pneumonia and meningitis¹⁴. This benefit may now be appreciated to arise not only by blocking bioactive PAF, but also by arresting the upshift of bacterial attachment leading to invasion of host cells. To demonstrate PAF-receptor-dependent translocation of virulent pneumococci *in vivo*, we tested the PAF-receptor antagonist for its ability to block activation-dependent adherence and transit of pneumococci from the alveolus into blood following intrapulmonary challenge in rabbit. Activation of the pulmonary epithelium with exogenous IL-1 increased the load of pneumococci recovered in lung lavage compared to unstimulated animals (Fig. 3; 6 hours). Adherence to IL-1 activated epithelium, progression to overt pneumonia, and nasal colonization were attenuated >90% by PAF-receptor antagonist (Fig. 3). All four control animals were bacteraemic at 48 hours compared with one of four treated with PAF-receptor antagonist (Fig. 3). In summary, we propose that presentation of the PAF receptor during inflammatory activation of host cells is critical to the biology of pneumococcal infection, acting by facilitating progression from colonization to bacteraemia. □

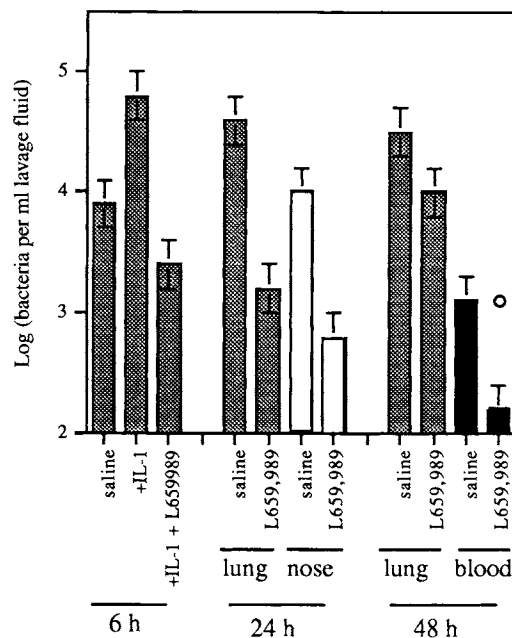


FIG. 3 Effect of PAF receptor antagonist on the course of pneumococcal infection *in vivo*. Number of pneumococci in bronchoalveolar lavage (grey bars), nasal lavage (white bars) or blood (black bars). One animal was bacteraemic in the L659,989 group at 48 h (circle); the bar represents mean CFU ml⁻¹ of the 3 others in this group.

METHODS. Rabbits ($n \geq 4$) were inoculated with 10^7 CFU ml⁻¹ type-2 pneumococcus All intratracheally or intranasally as described^{14,22}. Pneumococci were inoculated with saline solutions of IL-1 (5 ng), PAF-receptor antagonist L659,989 (130 μ g) or IL-1 plus L659,989. Animals from which samples were taken at 48 h received an additional intratracheal dose of L659,989 at 24 h.

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Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes

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In *Xenopus* oocytes, as well as other cells, inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃)-induced Ca²⁺ release^{1–4} is an excitable process that generates propagating Ca²⁺ waves^{5–7} that annihilate upon collision^{8–12}. The fundamental property responsible for excitability¹³ appears to be the Ca²⁺ dependency of the Ins(1,4,5)P₃ receptor⁹. Here we report that Ins(1,4,5)P₃-induced Ca²⁺ wave activity is strengthened by oxidizable substrates that energize mitochondria, increasing Ca²⁺ wave amplitude, velocity and interwave period. The effects of pyruvate/malate are blocked by ruthenium red at the Ca²⁺ uniporter, by rotenone at complex I, and by antimycin A at complex III, and are subsequently rescued at complex IV by ascorbate tetramethylphenylenediamine (TMPD)¹⁴. Our data reveal that potential-driven mitochondrial Ca²⁺ uptake is a major factor in the regulation of Ins(1,4,5)P₃-induced Ca²⁺ release and clearly demonstrate a physiological role of mitochondria in intracellular Ca²⁺ signalling.

Mitochondrial Ca²⁺ uptake is a passive process driven by the proton electrochemical gradient across the inner membrane¹⁵. Substrate oxidation increases the proton electrochemical gradient by increasing mitochondrial respiration¹⁴. To test whether mitochondrial Ca²⁺ uptake modulates Ins(1,4,5)P₃-induced Ca²⁺ release, we injected respiratory substrates into albino *Xenopus laevis* oocytes (stages V–VI). Ins(1,4,5)P₃-induced Ca²⁺ wave activity was assayed as described previously¹². Oocytes were injected with saturating concentrations (~10 mM final concentration) of either pyruvate/malate or succinate, 1–2 minutes after regenerative pulsatile Ca²⁺ wave activity was established by injecting inositol-1,4,5-trisphosphorothioate (IP₃S₃; ~5 μM final concentration). Oxidizable substrates rapidly changed the spatiotemporal pattern of Ca²⁺ release. Sequential images of a representative Ca²⁺ response show a significant reduction in the frequency of Ca²⁺ waves (Fig. 1). The number of pulsatile foci of Ca²⁺ release also decreased (Fig. 1a). In those foci which remain active, the interwave period increased from 9.8 ± 5.3 s to 51.0 ± 15.0 s (mean ± s.d., *n* = 5, *P* < 0.005) after substrate injection. The Ca²⁺ waves initiated from the remaining foci exhibit a highly continuous wavefront edge with an increased wave width as compared to controls (Fig. 1a). Moreover, Ca²⁺ waves have increased amplitude (Δ*F*/*F*), 0.45 ± 0.04 compared to control values of 0.21 ± 0.04 (*n* = 5, *P* < 0.05), and propagate at higher velocities, 33.2 ± 5.1 μm s⁻¹ compared to controls, 21.3 ± 3.4 μm s⁻¹ (*n* = 5, *P* < 0.05). We refer to this change in Ca²⁺ wave activity as a synchronization of Ca²⁺ release. Modulation of Ins(1,4,5)P₃ signalling by pyruvate/malate could be completely reversed by injection of rotenone, a specific respiratory chain inhibitor that blocks electron transport at Complex I (ref. 14). Ca²⁺ wave activity was first established in oocytes by co-injecting IP₃S₃ and pyruvate/malate. Subsequently, injection of rotenone reversed Ca²⁺ wave activity to control values in wave amplitude (0.58 ± 0.04 to 0.26 ± 0.05, *n* = 5, *p* < 0.025), interwave period (39.6 ± 9.0 s to 10.6 ± 2.3 s, *n* = 5, *p* < 0.005) and wave velocity (32.2 ± 4.4 μm s⁻¹ to 22.3 ± 2.1 μm s⁻¹, *n* = 5, *p* < 0.005) (Fig. 1d, e). Moreover, we tested the effects of antimycin A (30 min, 2 μg ml⁻¹), an inhibitor of electron transport through the respiratory chain between cytochrome *b* and cytochrome *c*₁ (complex III)¹⁴. This inhibition occurs after pyruvate/malate and succinate feed electrons into the respiratory chain. Oocytes treated with antimycin A were much less responsive to Ins(1,4,5)P₃ stimulation, whether injected alone or in combination with pyruvate/malate or succinate. A significantly smaller number of oocytes (31 of 54, 57%) were stimulated to release Ca²⁺ by Ins(1,4,5)P₃ injections as compared to controls (149 of 168 responding, 89%). Additionally, regenerative Ca²⁺ activity was rarely observed (6 of 31 regenerative, 19%) compared to control oocytes injected with either Ins(1,4,5)P₃ alone (89 of 149 regenerative, 60%) or Ins(1,4,5)P₃ concurrent with substrates (53 of 57 regenerative, 93%). We injected the artificial electron donor ascorbate/TMPD into oocytes treated with antimycin A (30 min, 2 μg ml⁻¹) to determine further whether these effects could be attributed to respiratory chain inhibition. Ascorbate/TMPD feeds electrons into the respiratory chain through cytochrome *c* oxidase (complex IV), beyond the point where antimycin A blocks electron transport¹⁴. Remarkably, addition of this electron donor rescued IP₃S₃-induced Ca²⁺ wave activity in antimycin A-treated oocytes (*n* = 8) (Fig. 2). Taken together, these data indicate that modulation of Ins(1,4,5)P₃ signalling by injection of oxidizable substrates is due to increased mitochondrial activity.

Substrate oxidation is predicted to increase the mitochondrial membrane potential. To monitor changes in mitochondrial membrane potential, oocytes were incubated with the potential sensitive dye tetramethyl rhodamine methyl ester (TMRM)¹⁶. Oocyte mitochondrial potentials were relatively low (92.6 ± 10.5 mV, *n* = 5) compared with the mitochondria of the enveloping follicular cells (157 ± 8.2 mV, *n* = 3). However, substrate injection significantly increased oocyte mitochondrial

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membrane potentials to 119 ± 10.4 mV ($n=5$, $p<0.005$) (Fig. 3), whereas antimycin A-treated oocytes exhibited a collapsed potential that was rescued by ascorbate/TMPD injections (data not shown). These data demonstrate that injection of oxidizable substrates increases mitochondrial potential, which in turn can stimulate ATP production or mitochondrial Ca^{2+} uptake.

We injected ATP into oocytes instead of oxidizable substrates to test whether ATP production mediated the effect of energized mitochondria. At a final concentration of 1 mM, ATP did not elicit any change in Ca^{2+} wave activity ($n=8$). Injection of higher ATP concentrations (2.5 mM, $n=6$; 5 mM, $n=5$; 10 mM, $n=10$; approximate final concentrations) produced a small transient rise in Ca^{2+} followed by complete inhibition of Ca^{2+} wave activity. During inhibition of wave activity, the cytosolic Ca^{2+} concentration was usually decreased below resting Ca^{2+} levels. Similar effects were observed when ATP- γ -S was injected (~ 10 mM final concentration, $n=5$), although the cytosolic Ca^{2+} concentration decreased more slowly after the initial rise.

Consistent with this, previous results¹⁷ have demonstrated that high concentrations of ATP (>4 mM) decrease $\text{Ins}(1,4,5)\text{P}_3$ receptor channel activity. Furthermore, modulation of Ca^{2+} wave activity by injection of pyruvate/malate was not affected by pretreatment of oocytes ($n=10$) with oligomycin ($10 \mu\text{g ml}^{-1}$, ~ 60 min bath incubation), a specific inhibitor of the mitochondrial ATP synthetase¹⁴. Taken together these data suggest that the effects of energized mitochondria on $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} wave activity cannot be accounted for by an increase in ATP production.

The mitochondrial Ca^{2+} uniporter is the potential driven electrogenic transport system that allows rapid sequestration of Ca^{2+} into mitochondria¹⁵. The magnitude of the mitochondrial potentials encountered in the oocyte is in the range where the rate of mitochondrial Ca^{2+} uptake is most sensitive to potential changes¹⁸. It is well established that the polycation ruthenium red inhibits the Ca^{2+} uniporter of mitochondria¹⁹. We tested whether this transport system was involved in $\text{Ins}(1,4,5)\text{P}_3$ -medi-

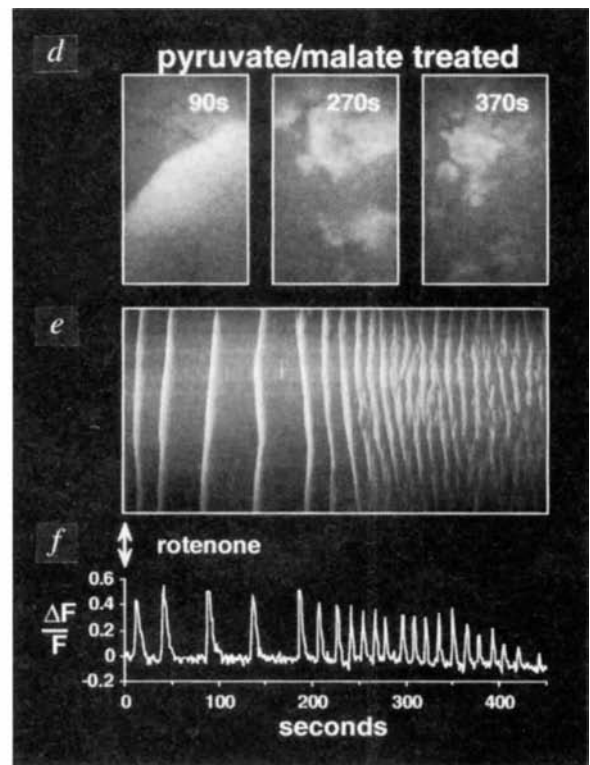
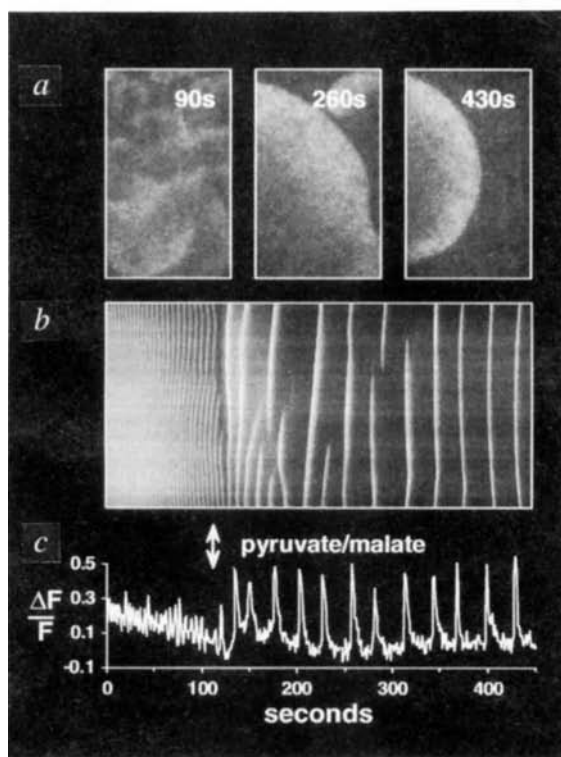


FIG. 1 Mitochondrial substrates synchronize IP_3S_3 -induced Ca^{2+} wave activity. Images in this oocyte are acquired 1 min after IP_3S_3 injection ($\sim 5 \mu\text{M}$ final concentration). **a**, Individual confocal images are $429 \times 726 \mu\text{m}$ (130×220 pixels). **b**, Temporal stack of the entire 450 images observed before and after pyruvate/malate injection (~ 10 mM final concentration; injected at arrow). The temporal image (450×220 pixels) was produced by stacking 450 individual images (1×220 pixels) edgewise. **c**, Time course of the average fluorescence intensity recorded from a 5×5 pixel area. **d-f**, Rotenone reverses the modulation of IP_3S_3 -induced Ca^{2+} wave activity by pyruvate/malate. Rotenone ($\sim 250 \mu\text{M}$ final concentration) is injected at 0 s (arrow), ~ 5 min after the oocyte was initially co-injected with IP_3S_3 ($\sim 5 \mu\text{M}$ final concentration) and pyruvate/malate (~ 10 mM final concentration). Rotenone inhibition of pyruvate/malate oxidation (complex I) becomes noticeable at ~ 200 s when Ca^{2+} wave frequency begins to increase and amplitude decreases to control values (see main text). **d**, Individual images before (90 s) and after (270 s, 370 s) rotenone reversal of pyruvate/malate treatment. **e**, Temporal stack of the entire 450 images. **f**, Time course of average fluorescence intensity for an arbitrary 5×5 pixel area. Intensity is expressed as $\Delta F/F$, which represents $(F_{\text{peak}} - F_{\text{rest}})/F_{\text{rest}}$. METHODS. Ca^{2+} was imaged as previously described¹². Briefly, oocytes

were assayed 24–72 h after surgical removal and defolliculation, and were injected 6–12 h before confocal imaging; each oocyte was injected with the Ca^{2+} dye indicator Ca^{2+} green dextran (relative molecular mass (M_r) 70K; Molecular Probes, Eugene, OR; 50 nl of 0.5 mg ml^{-1}). Images (170×170 pixels or 256×256 pixels) were acquired at 1-s intervals with a BioRad MRC 600UV confocal laser scanning microscope at $\times 1.5$ attached to a Nikon Diaphot with a $\times 10$ (0.5 NA) UVfluor Nikon objective lens using Time Course Ratiometric Software (TCSM, BioRad). The confocal aperture was set at the largest opening, producing a $745 \times 745 \times 32 \mu\text{m}$ optical slice. All recordings were made in a medium containing 96 mM NaCl, 2 mM KCl, 2 mM MgCl_2 , 5 mM HEPES, pH 7.5 (Gibco, Grand Island, NY). Substrates were prepared as Tris salts (~ 10 mM final concentration, pH 7.0) (Sigma, St Louis, MO). Images were processed with ANALYZE software (Mayo Foundation, Rochester, MN) on a Sun Sparc2 workstation. Figures were filtered with a 5×5 sigma filter (edge-preserving low-pass filter). Rotenone was solubilized in dimethyl sulphoxide (DMSO) (Sigma) and injected as a 9 nl bolus. Control oocytes injected only with DMSO showed no change in Ca^{2+} wave activity ($n=5$). All other injections are delivered as a 50 nl bolus, so that a final concentration represents a 1:20 dilution, assuming a $1 \mu\text{l}$ oocyte volume.

ated Ca^{2+} release by preinjecting oocytes with ruthenium red ($25 \mu\text{M}$ for 1 h). The effects of oxidizable substrates on regenerative Ca^{2+} wave activity were abolished in pretreated oocytes. These oocytes exhibited an interwave period of $9.2 \pm 3.9 \text{ s}$ ($n=6$) and a wave amplitude ($\Delta F/F$) of 0.26 ± 0.11 , values indistinguishable from oocytes injected with IP_3S_3 alone (9.8 s and 0.21 , see above). Another common target of ruthenium red in many cells is the ryanodine receptor¹. Preinjection of oocytes ($n=8$) with ryanodine ($20 \mu\text{M}$ for 1 h) did not affect the action of the respiratory chain substrates, a finding consistent with *Xenopus* oocytes appearing not to have ryanodine receptors²⁰. However, heparin ($50 \mu\text{M}$), a competitive inhibitor of the $\text{Ins}(1,4,5)\text{P}_3$ receptor, completely blocked Ca^{2+} release when injected during wave activity in the presence of pyruvate/malate or succinate ($n=6$). We interpret these data as evidence that the action of mitochondria on $\text{Ins}(1,4,5)\text{P}_3$ -mediated Ca^{2+} wave activity is dependent on the operation of a mitochondrial ruthenium red-sensitive Ca^{2+} uniporter.

Mitochondrial studies indicate that the Ca^{2+} uniporter is significantly activated only at Ca^{2+} concentrations above 500 nM , indicating that the uniporter has a much lower affinity for Ca^{2+} than sarco-endoplasmic reticulum Ca^{2+} -ATPases (SERCAs)^{15,19}. These findings predict that SERCAs should compete with mitochondria for Ca^{2+} in the cytoplasm, thereby muting the effects of respiratory chain substrates on $\text{Ins}(1,4,5)\text{P}_3$ -

mediated signalling. To test this hypothesis, oocytes were injected with avian SERCA1 messenger RNA, as previously described¹², and assayed for Ca^{2+} activity 4–7 days later. As reported, expression of SERCA1 increases the frequency of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} waves (Fig. 4). However, injection of oxidizable substrates into these SERCA1-expressing oocytes was ineffective at altering Ca^{2+} wave activity. Injection of pyruvate/malate or succinate during regenerative Ca^{2+} wave activity did not change the Ca^{2+} wave period, $3.2 \pm 0.7 \text{ s}$ compared to $3.5 \pm 1.0 \text{ s}$ ($n=10$) after treatment. A slight decrease in wave amplitude was observed in these oocytes after addition of oxidizable substrates, from control values of 0.49 ± 0.12 to 0.40 ± 0.16 ($n=10$, not significant). We conclude from these data that increased Ca^{2+} ATPase activity can successfully compete with the substrate-enhanced ability of mitochondria to sequester Ca^{2+} . Additionally, they suggest that the contribution of mitochondria to Ca^{2+} signalling in any cell type will depend on the relative density of mitochondria and SERCAs, even when substrate supply is not limited and the contribution of mitochondrial Ca^{2+} uptake remains relatively constant.

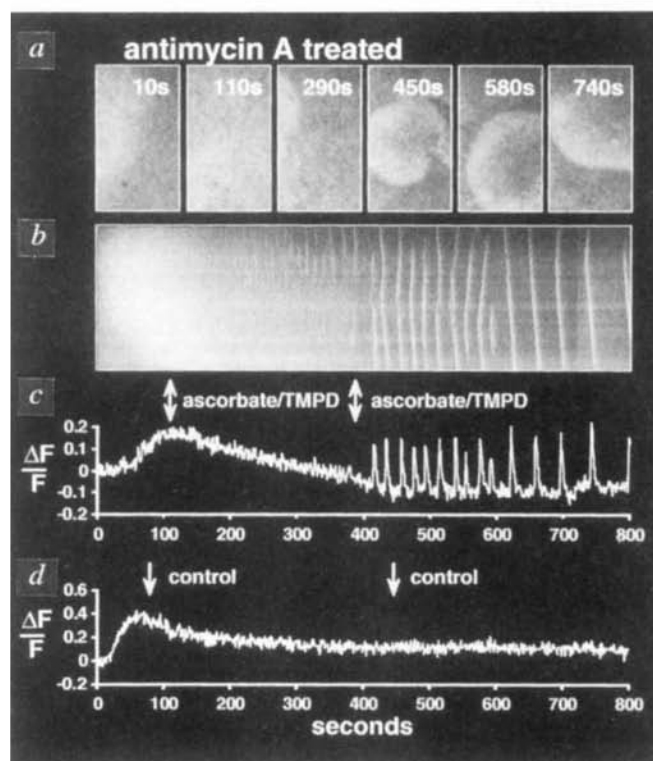


FIG. 2 Ascorbate/TMPD restoration of IP_3S_3 -induced Ca^{2+} wave activity in antimycin A-treated oocytes. **a**, Individual images of IP_3S_3 ($\sim 5 \mu\text{M}$ final concentration) induced Ca^{2+} release in an oocyte treated with antimycin A ($2 \mu\text{g ml}^{-1}$, for 30 min). IP_3S_3 was injected at 0 s. **b**, Temporal stack of the entire 800 images, displayed as in previous figures. Regenerative Ca^{2+} wave activity is visible only after substrate injection (arrows) of ascorbate/TMPD ($\sim 5 \text{ mM}/0.5 \text{ mM}$ in 10 mM Tris, final concentration, pH 7.0). Note that the first injection produced a partial recovery of regenerative activity. After $\sim 5 \text{ min}$, a second ascorbate/TMPD injection produced stronger wave activity throughout the entire imaging field. **c**, Corresponding time course of the average Ca^{2+} fluorescence intensity ($\Delta F/F$) recorded in an arbitrary 5×5 pixel area. **d**, Another antimycin A-treated oocyte that was injected with control buffer only (10 mM Tris (final concentration) pH 7.0).

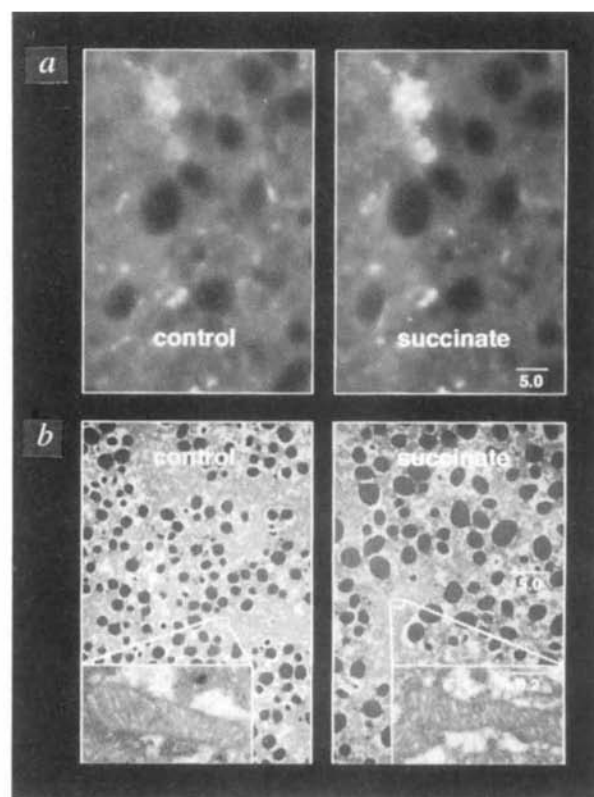


FIG. 3 Substrate-induced increase in mitochondrial membrane potential. **a**, TMRM ($\sim 100 \text{ nM}$) images of an oocyte before (control) and 10 min after substrate injection (succinate). **b**, Electron micrographs show control oocytes and succinate injected oocytes (fixed 10 min after injection). Insets show enlargements of mitochondria within clusters. Mitochondria have previously been reported to cluster within corridors of yolk-free cytoplasm in *Xenopus* oocytes³⁰. **METHODS**. Oocytes were incubated with 600 nM TMRM for 20 min and then bathed in 100 nM TMRM for the duration of the recording¹⁶. Fluorescence images were collected from one slow confocal scan with a $\times 100$ UVFluor Nikon oil, 1.3 NA objective, and low-pass filtered (5×5 sigma filter). For electron micrographs, oocytes were fixed in phosphate-buffered 4% paraformaldehyde and 5% glutaraldehyde for 24 h, post-fixed in 2% osmium tetroxide, dehydrated in acetone, and embedded in Epon 812 (Ernest F. Fulham, Latham, NY). Semithin sections were cut from tissue blocks and stained with toluidine blue. The blocks were oriented tangential to the plasma membrane (coinciding with confocal images), trimmed and sectioned for electron microscopy. Thin sections were stained with uranyl acetate and lead citrate, and then observed and photographed in a JEOL 100CX electron microscope.

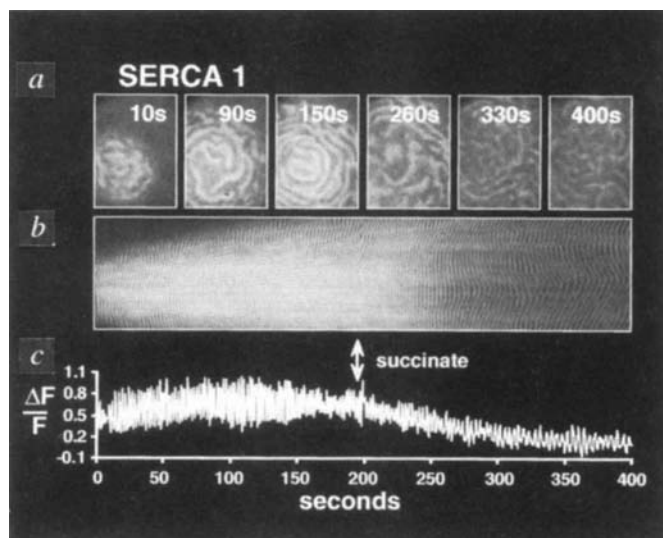


FIG. 4 Increased SERCA 1 expression overpowers the action of oxidizable substrates on Ca^{2+} wave activity. SERCA1 messenger RNA was expressed as previously described¹². *a*, Individual images of $\text{Ins}(1,4,5)\text{P}_3$ (~300 nM final concentration)-induced Ca^{2+} wave activity in a SERCA1-expressing oocyte before and after injection of succinate (~10 mM in 10 mM Tris (final concentration) pH 7.0, arrow). *b*, Temporal stack of images taken at 0.5 s intervals. *c*, Time course of average fluorescence intensity ($\Delta F/F$) for an arbitrary 5×5 pixel area.

$\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} wave activity in *Xenopus* oocytes can be characterized as an excitable medium⁸ in which Ca^{2+} -induced Ca^{2+} release (CICR) of the bound $\text{Ins}(1,4,5)\text{P}_3$ receptor constitutes the autocatalytic process that generates the elementary excitable event⁹. The threshold for excitation is defined as the Ca^{2+} concentration at which CICR begins to raise cytosolic Ca^{2+} faster than Ca^{2+} buffering systems remove it. We propose that Ca^{2+} uptake into energized mitochondria will raise the threshold, increasing the amount of Ca^{2+} stimulus needed for the autocatalytic rise in cytosolic Ca^{2+} , and thereby decrease wave frequency. A higher threshold also decreases the number of asynchronous $\text{Ins}(1,4,5)\text{P}_3$ receptor channel openings, because most channels have insufficient Ca^{2+} stimulus to open. As a consequence, a single propagating Ca^{2+} wave will excite more $\text{Ins}(1,4,5)\text{P}_3$ receptors, increasing wave amplitude and sharpening the leading edge of the wavefront. We refer to this process as mitochondrial-generated synchronization of Ca^{2+} release. The increased density of excitable $\text{Ins}(1,4,5)\text{P}_3$ receptors also accounts for increased propagation velocities, owing to the shorter Ca^{2+} diffusion distance between excitable receptors. In addition to raising the threshold for Ca^{2+} excitation, mitochondrial Ca^{2+} uptake may also contribute to slowing ion channel inactivation by reducing the maximal Ca^{2+} concentration in the immediate vicinity of the bound $\text{Ins}(1,4,5)\text{P}_3$ receptor²¹. This could account for the inhibition of regenerative Ca^{2+} wave activity in the presence of antimycin A, which eliminates mitochondrial Ca^{2+} buffering. Given the low *in vitro* Ca^{2+} sensitivity of mitochondria, the action of substrates on Ca^{2+} signalling may be due in part to a close proximity of mitochondria and $\text{Ins}(1,4,5)\text{P}_3$ receptors^{22–24}, because local concentrations of Ca^{2+} are much higher near the channel pore. It has also been shown that mitochondria sequester Ca^{2+} during receptor-mediated intracellular Ca^{2+} release^{25–28}. Irrespective of the detailed mechanism, we conclude that potential-driven Ca^{2+} uptake by mitochondria, in addition to Ca^{2+} ATPases¹², is a major factor in modulating $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release. Physiological factors that affect the respiratory state of mitochondria, as well as unique morphological arrangements between mitochondria and endoplasmic reticulum²⁹, will need to be reexamined in the light of

this new role of mitochondria in the dynamics of $\text{Ins}(1,4,5)\text{P}_3$ -mediated intracellular Ca^{2+} signalling. □

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Control of p70 S6 kinase by kinase activity of FRAP *in vivo*

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WHEN complexed with the intracellular protein FKBP12, rapamycin is a potent immunosuppressant^{1,2} and an inhibitor of a mitogen-stimulated signalling pathway that leads to activation of p70 S6 kinase^{3–6} (p70^{S6k}) and cyclin-dependent kinases^{7–10} (CDKs). A recently cloned FKBP12–rapamycin-associated protein (FRAP/RAFT) is the likely mediator of these effects^{11,12}. Using FRAP variants that do not bind FKBP12–rapamycin, we demonstrate here that FRAP is a rapamycin-sensitive regulator of p70^{S6k} *in vivo* and that the kinase activity of FRAP is required for this regulation. In addition, we show that FRAP autophosphorylates *in vitro*. Consistent with an essential role for FRAP kinase activity *in vivo*, autophosphorylation of FRAP is inhibited by FKBP12–rapamycin. Deletion studies indicate that the kinase activity of FRAP alone is not sufficient for control of p70^{S6k} and that an amino-terminal domain in FRAP is also required.

The putative kinase domain of FRAP, which has homology to phosphatidylinositol (PtdIns) kinases^{11,12}, is located in the carboxy terminus and is thought to have phosphoryltransferase activity because of its identity with residues necessary for ATP binding and γ -PO₄ transfer in PtdIns and protein kinases^{13–15}. In order to serve as negative controls in an investigation of FRAP kinase function, two aspartic acid residues in FRAP that

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10-fold less susceptible to increasing concentrations of rapamycin treatment compared to HA-p70^{S6k} expressed alone (Fig. 3b). This shift in dose-response is consistent with a role for wild-type FRAP in regulating p70^{S6k} activity *in vivo* and is analogous to a similar shift observed in response to FK506 upon expression of calcineurin in the same cell line¹⁶. Together these data indicate that FRAP is a rapamycin-sensitive regulator of p70^{S6k} activity *in vivo*.

The rapamycin-resistant phenotype produced by expression of the S2,035 FRAP variants was not observed when these proteins also contained either the D2,338A or the D2,357E mutation (Fig. 3a,b). HA-p70^{S6k} activity was completely inhibited by rapamycin treatment in cells expressing the S2,035T/D2,338A, S2,035T/D2,357E, S2,035I/D2,338A, or the S2,035I/D2,357E FRAP mutant (Fig. 3a). Susceptibility of HA-p70^{S6k} to a range of rapamycin doses in cells expressing S2,035T/D2,357E FRAP was identical to that of cells expressing HA-p70^{S6k} alone (Fig. 3b). Indicative of dephosphorylation and inactivation^{4,18}, the characteristic increase in p70^{S6k} mobility following rapamycin treatment³⁻⁶ paralleled the activity of immunoprecipitated HA-p70^{S6k} (Fig. 3c). HA-p70^{S6k} was resistant to dephosphorylation following rapamycin treatment in cells expressing the S2,035 variants but was susceptible in cells expressing the S2,035 variants containing a mutation at D2,338 or D2,357. These data

indicate that the kinase function in FRAP is necessary for regulating p70^{S6k} activity *in vivo*.

Consistent with an essential role for FRAP kinase activity *in vivo*, *in vitro* autophosphorylation of FRAP expressed in either Tag Jurkat or Sf9 cells was specifically inhibited by FKBP12-rapamycin but not by FKBP12 alone or by a complex of FKBP12 and FK506 (Fig. 3d). The potency of FKBP12-rapamycin as an inhibitor of autophosphorylation (IC_{50} = 10–15 nM FKBP12-rapamycin; data not shown) is similar to the previously reported high-affinity interaction between FKBP12-rapamycin and the 11K FKBP12-rapamycin binding (FRB) domain of FRAP (ref. 17). In addition, concordant with *in vivo* evidence suggesting that the endogenous rapamycin-sensitive regulator of p70^{S6k} activity is resistant to the PtdIns-3-OH kinase inhibitor wortmannin¹⁹, autophosphorylation of FRAP was not abolished by concentrations of wortmannin that are 25- and 250-fold greater than its IC_{50} for inhibition of PtdIns-3-OH kinase (Fig. 3d). Thus it is likely that *in vivo*, FKBP12-rapamycin exerts its effects on p70^{S6k} and other rapamycin-sensitive molecules such as cdk^s⁷⁻⁹ and p27^{kip} (ref. 10) by inhibiting the intrinsic kinase activity of FRAP, possibly in a substrate-specific manner²⁰.

In order to determine whether the FRAP kinase domain alone is sufficient for control of p70^{S6k} activity, truncations were made in the N-terminal coding sequence of FRAP. As assessed by the

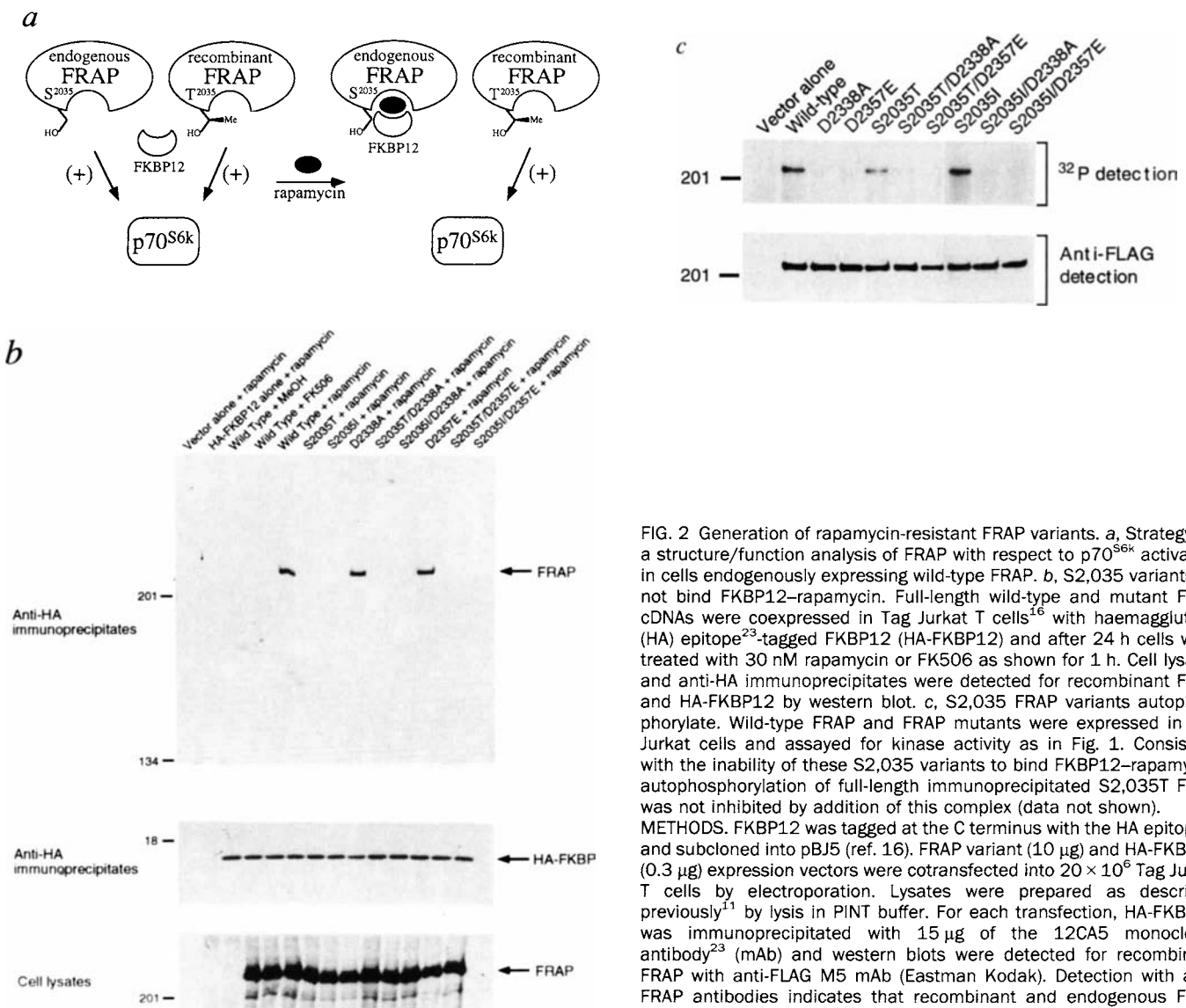


FIG. 2 Generation of rapamycin-resistant FRAP variants. **a**, Strategy for a structure/function analysis of FRAP with respect to p70^{S6k} activation in cells endogenously expressing wild-type FRAP. **b**, S2,035 variants do not bind FKBP12-rapamycin. Full-length wild-type and mutant FRAP cDNAs were coexpressed in Tag Jurkat T cells¹⁶ with haemagglutinin (HA) epitope²³-tagged FKBP12 (HA-FKBP12) and after 24 h cells were treated with 30 nM rapamycin or FK506 as shown for 1 h. Cell lysates and anti-HA immunoprecipitates were detected for recombinant FRAP and HA-FKBP12 by western blot. **c**, S2,035 FRAP variants autophosphorylate. Wild-type FRAP and FRAP mutants were expressed in Tag Jurkat cells and assayed for kinase activity as in Fig. 1. Consistent with the inability of these S2,035 variants to bind FKBP12-rapamycin, autophosphorylation of full-length immunoprecipitated S2,035T FRAP was not inhibited by addition of this complex (data not shown). **METHODS.** FKBP12 was tagged at the C terminus with the HA epitope²³ and subcloned into pBJ5 (ref. 16). FRAP variant (1.0 µg) and HA-FKBP12 (0.3 µg) expression vectors were cotransfected into 20×10^6 Tag Jurkat T cells by electroporation. Lysates were prepared as described previously¹¹ by lysis in PINT buffer. For each transfection, HA-FKBP12 was immunoprecipitated with 15 µg of the 12CA5 monoclonal antibody²³ (mAb) and western blots were detected for recombinant FRAP with anti-FLAG M5 mAb (Eastman Kodak). Detection with anti-FRAP antibodies indicates that recombinant and endogenous FRAP comigrate during electrophoresis (data not shown).

HA-p70^{S6k} coexpression assay described in Fig. 3, the ability of S2,035T FRAP to render cells resistant to rapamycin was diminished 90% by deletion of the N-terminal 91 amino acids of FRAP ($\Delta 1-91$) (Fig. 4a). Resistance to rapamycin was abolished by further N-terminal truncation ($\Delta 1-500$ and $\Delta 1-909$). Again, the indicative shift in p70^{S6k} mobility following rapamycin treatment (Fig. 4b) paralleled the activity of immunoprecipitated p70^{S6k} activity (Fig. 4a). Deletion of the N terminus,

however, did not affect either *in vitro* autophosphorylation (Fig. 4c) or binding to FKBP12-rapamycin (Fig. 4d), indicating that the structural integrity of the C-terminal region of FRAP remains intact. These results indicate that, in addition to a functional kinase domain, a region encoded within the N terminus of FRAP is required for regulating p70^{S6k} activity *in vivo*. Although it is possible that the N terminus may bind p70^{S6k}, we have as yet been unable to detect either p70^{S6k} or any specific

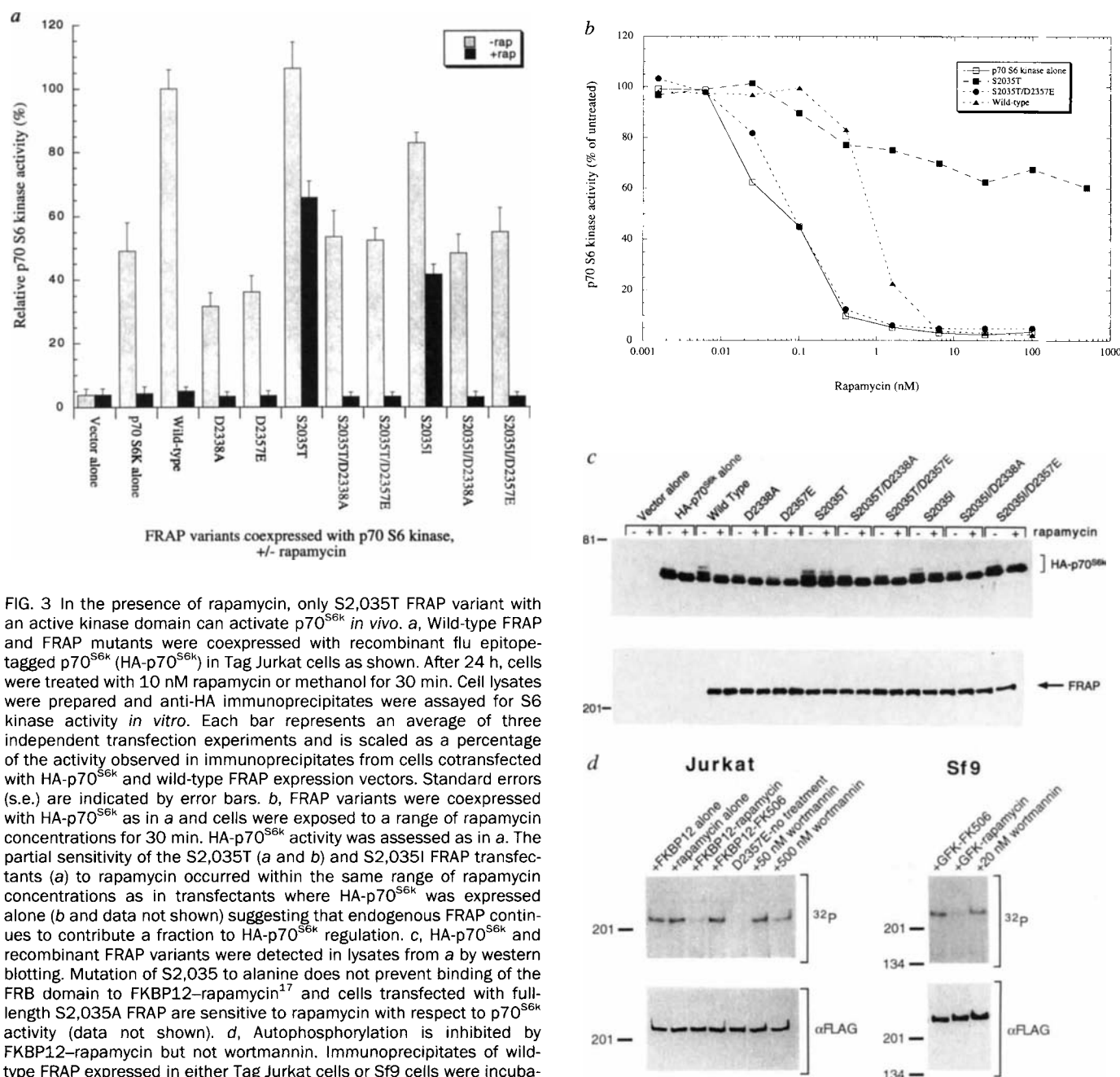


FIG. 3 In the presence of rapamycin, only S2,035T FRAP variant with an active kinase domain can activate p70^{S6k} *in vivo*. **a**, Wild-type FRAP and FRAP mutants were coexpressed with recombinant flu epitope-tagged p70^{S6k} (HA-p70^{S6k}) in Tag Jurkat cells as shown. After 24 h, cells were treated with 10 nM rapamycin or methanol for 30 min. Cell lysates were prepared and anti-HA immunoprecipitates were assayed for S6 kinase activity *in vitro*. Each bar represents an average of three independent transfection experiments and is scaled as a percentage of the activity observed in immunoprecipitates from cells cotransfected with HA-p70^{S6k} and wild-type FRAP expression vectors. Standard errors (s.e.) are indicated by error bars. **b**, FRAP variants were coexpressed with HA-p70^{S6k} as in **a** and cells were exposed to a range of rapamycin concentrations for 30 min. HA-p70^{S6k} activity was assessed as in **a**. The partial sensitivity of the S2,035T (**a** and **b**) and S2,035I FRAP transfectants (**a**) to rapamycin occurred within the same range of rapamycin concentrations as in transfectants where HA-p70^{S6k} was expressed alone (**b** and data not shown) suggesting that endogenous FRAP continues to contribute a fraction to HA-p70^{S6k} regulation. **c**, HA-p70^{S6k} and recombinant FRAP variants were detected in lysates from a by western blotting. Mutation of S2,035 to alanine does not prevent binding of the FRB domain to FKBP12-rapamycin¹⁷ and cells transfected with full-length S2,035A FRAP are sensitive to rapamycin with respect to p70^{S6k} activity (data not shown). **d**, Autophosphorylation is inhibited by FKBP12-rapamycin but not wortmannin. Immunoprecipitates of wild-type FRAP expressed in either Tag Jurkat cells or Sf9 cells were incubated with preformed complexes of FKBP12 or GST-FKBP12 (GFK, ref. 11) and the indicated compounds at a concentration of 500 nM before addition of γ -³²P ATP.

METHODS. HA-p70^{S6k} is a fusion of the HA epitope²³ with the N terminus of p70^{S6k} (ref. 24) and expression was driven by the pBJ5 vector¹⁶. For each transfection, 8 μ g FRAP variant and 50 ng HA-p70^{S6k} expression vectors were transfected into 20×10^6 Tag Jurkat cells by electroporation. Lysis was as described in Fig. 2. For immunoprecipitation of HA-p70^{S6k}, 5 μ g of 12CA5 mAb was added to clarified lysates and complexed to protein A beads (Gibco/BRL). Complexes were then washed three times with lysis buffer and once with kinase reaction buffer²⁵ containing 1 mM DTT. Immunoprecipitates were then incubated

at 37 °C for 20 min in kinase reaction buffer containing 50 μ M KRRRLASLAA peptide (ref. 26) as substrate, 100 μ M γ -³²P ATP (0.5 Ci mmol⁻¹) and 1 U μ l⁻¹ PKI (Sigma). Reactions were then spotted onto P81 paper as described²⁶. On average HA-p70^{S6k} incorporated 3.44 ± 0.85 (s.e.) pmol of phosphate into substrate per min when isolated from cells cotransfected with HA-p70^{S6k} and wild-type FRAP expression vectors. Western blots were detected either with the 12CA5 mAb or the anti-FLAG M5 mAb. Autophosphorylation of FRAP immunoprecipitates were as in Fig. 1 except immunocomplexes were incubated with the complexes/compounds indicated for 30 min at 4 °C before a 5 min incubation at 30 °C with [γ -³²P]ATP.

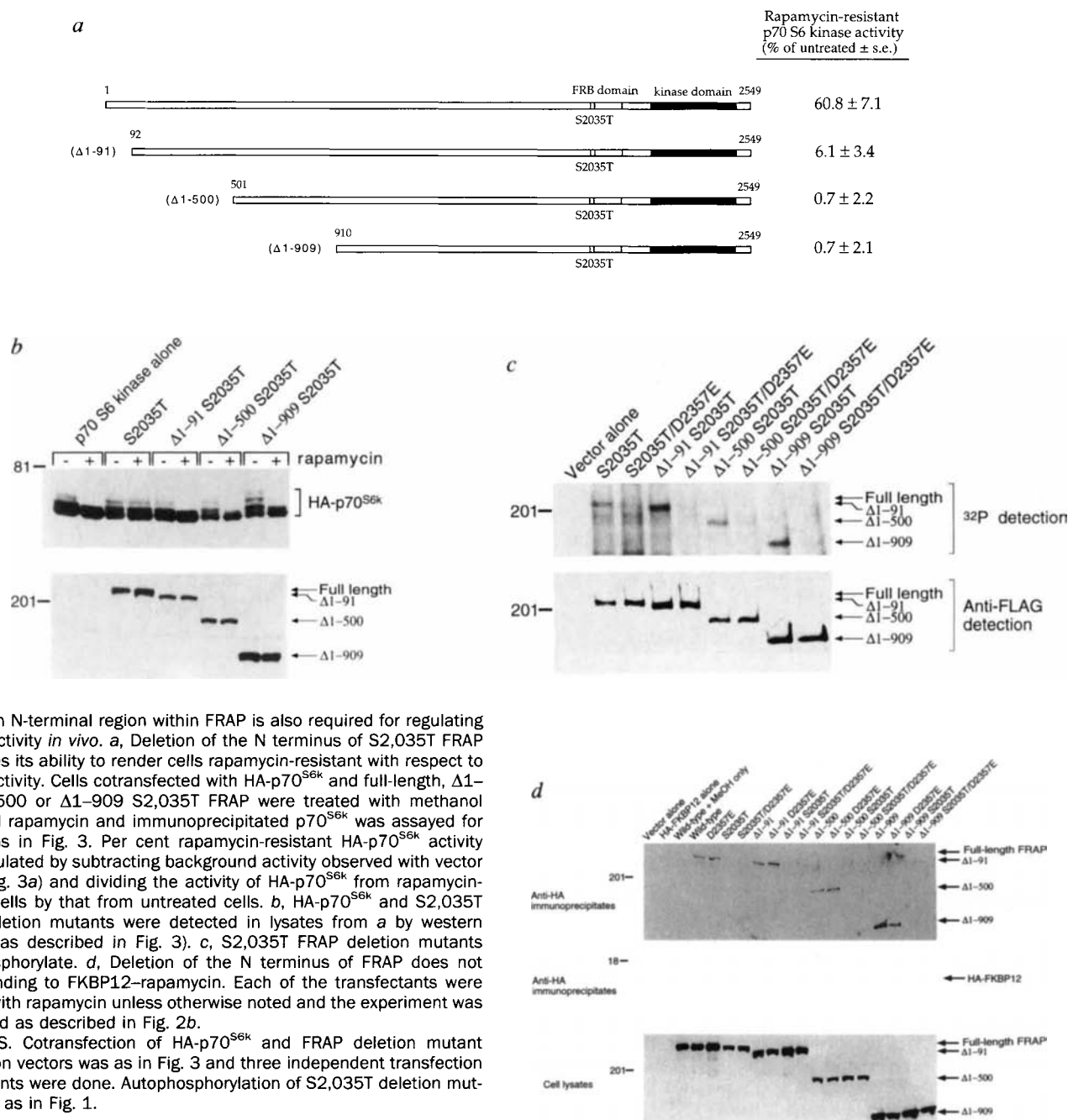


FIG. 4 An N-terminal region within FRAP is also required for regulating p70^{S6k} activity *in vivo*. **a**, Deletion of the N terminus of S2,035T FRAP eliminates its ability to render cells rapamycin-resistant with respect to p70^{S6k} activity. Cells cotransfected with HA-p70^{S6k} and full-length, Δ 1-91, Δ 1-500 or Δ 1-909 S2,035T FRAP were treated with methanol or 10 nM rapamycin and immunoprecipitated p70^{S6k} was assayed for activity as in Fig. 3. Per cent rapamycin-resistant HA-p70^{S6k} activity was calculated by subtracting background activity observed with vector alone (Fig. 3a) and dividing the activity of HA-p70^{S6k} from rapamycin-treated cells by that from untreated cells. **b**, HA-p70^{S6k} and S2,035T FRAP deletion mutants were detected in lysates from **a** by western blotting (as described in Fig. 3). **c**, S2,035T FRAP deletion mutants autophosphorylate. **d**, Deletion of the N terminus of FRAP does not affect binding to FKBP12-rapamycin. Each of the transfectants were treated with rapamycin unless otherwise noted and the experiment was performed as described in Fig. 2b. **METHODS.** Cotransfection of HA-p70^{S6k} and FRAP deletion mutant expression vectors was as in Fig. 3 and three independent transfection experiments were done. Autophosphorylation of S2,035T deletion mutants was as in Fig. 1.

S6 kinase activity in wild-type or mutant FRAP immunoprecipitates (data not shown). Further definition of this N-terminal domain will aid investigation of its involvement in regulating p70^{S6k} activity.

This study suggests that two domains in FRAP are required for regulating p70^{S6k} activity *in vivo*. Both the ability of FKBP12-rapamycin to inhibit autophosphorylation *in vitro* and the inability of S2,035 variants containing kinase inactivating mutations to signal *in vivo* indicate an obligatory role for the kinase activity of FRAP in regulating p70^{S6k}. Given that autophosphorylation of FRAP is inhibited by FKBP12-rapamycin, it is possible that autophosphorylation itself may be the rapamycin-sensitive step towards signalling to downstream effector molecules. This study also illustrates a chemical/genetic approach to the dissection of protein function *in vivo*. Despite ubiquitous expression of FRAP (ref. 11), a structure/function analysis was made possible by the combination of a rapamycin-resistant form of FRAP and rapamycin itself. By analogy, inhibi-

tion of FRAP by rapamycin can be viewed as a chemical equivalent to a loss of function conditional allele of endogenous FRAP. A similar strategy may be useful in the study of other proteins whose functions are sensitive to cell-permeable compounds. □

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Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B

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CYTOTOXIC T lymphocyte (CTL)-mediated cytotoxicity represents the body's major defence against virus-infected and tumorigenic cells, and contributes to transplant rejection and autoimmune disease. During killing, CTL granules are exocytosed, releasing their contents into the intercellular space between the target cell and the effector. Perforin facilitates the entry of cytotoxic cell serine proteases, the granzymes, into the target cell, where they induce apoptotic death by an unknown pathway¹. Granzyme B is essential for the induction of DNA fragmentation and apoptosis in target cells^{2–5}, yet its substrate is unknown. Identification of the intracellular substrate for granzyme B is therefore the key to understanding the mechanism of CTL-mediated killing. Here we show that granzyme B cleaves and activates CPP32, the precursor of the protease responsible for cleavage of poly(ADP-ribose) polymerase.

Each of the ICE/*ced-3* proteases, which are involved in apoptosis (see ref. 6), is synthesized as an inactive precursor requiring cleavage at specific Asp residues to produce two subunits of relative molecular mass (*M_r*) 20K and 10K, which together form the active protease. Because the cleavages that occur during ICE/*ced-3* protease activation are at Asp-X bonds, they are excellent potential substrates for granzyme B (ref. 7), which has substrate specificity requiring an Asp at P₁ (refs 8–10). We have previously shown that ICE itself is not a granzyme B substrate¹¹, but other ICE/*ced-3* proteases may be more important for the induction of apoptosis than ICE itself¹². Hence we investigated Apoptain/CPP32, which was isolated on the basis of its ability to cleave poly(ADP-ribose)polymerase (PARP) during the induction of apoptosis¹³. Cleavage of PARP, an enzyme involved in DNA repair and genome maintenance^{14,15}, may lead to inhibition of DNA repair processes as well as the

release of negative regulation of the Ca²⁺/Mg²⁺-dependent endonuclease involved in DNA fragmentation, a hallmark of apoptosis¹⁶. PARP may also contribute to cell death by depletion of NAD and ATP in the cell¹⁷. PARP is not essential, however, as PARP knockout mice develop normally but are more susceptible to environmental stress¹⁸. Thus, although PARP cleavage may act as a marker for CPP32 activity, it seems likely that CPP32 induces apoptosis by cleaving several proteins.

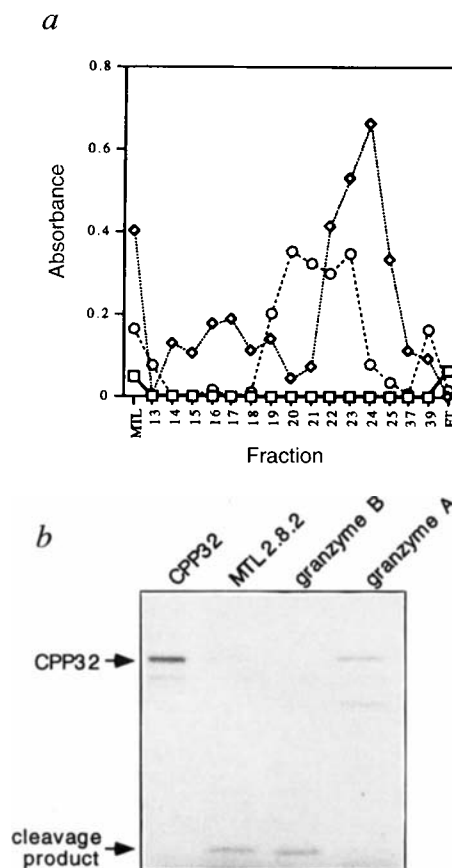


FIG. 1 Cleavage of CPP32 by purified granzyme B. **a**, Enzymatic activity of FPLC fractions from MTL 2.8.2 cell lysate. MTL, MTL 2.8.2 lysate before purification; FT, flow-through fraction from purification. **b**, Cleavage of CPP32 by whole MTL 2.8.2 lysate, FPLC fraction 20 (granzyme B), FPLC fraction 24 (granzyme A). [³⁵S]CPP32 alone was used as a negative control. Symbols represent the following synthetic substrates: circles, Asp-ase (*tert*-Boc-AAD-Bz); diamonds, BLT Esterase (*N*α-CBZ-L-Lys-Bz); squares, Chym-ase (Succ-AAPFS-Bz).

METHODS. **a**, Granzyme B was separated from MTL 2.8.2 cell lysate using a modification of a method described previously²². Briefly, MTL 2.8.2 cells were lysed in 1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, and then made to a final concentration of 390 mM NaCl followed by FPLC over a Mono-S column. Of each fraction, 10 μl was assayed^{9,10,17} for enzymatic activities with the synthetic substrate indicated. **b**, CPP32 was cloned by polymerase chain reaction (PCR) using primers corresponding to the first 30 nucleotides (forward) and last 31 nucleotides (reverse) of the open reading frame of CPP32-β (accession no. U13738) that were synthesized to contain 5' *Bam*HI, *Xho*I and *Xba*I restriction sites. Sequence-verified products in the *Xba*I site of pBluescript II SK+ (Stratagene) were used to drive the synthesis of [³⁵S]Met-labelled CPP32 by coupled transcription/translation (Promega). Translation mixes were purified by gel permeation FPLC to remove unincorporated [³⁵S]methionine and constituents of the reticulocyte lysate. Of each FPLC fraction from the granzyme separation, 500 μl was concentrated to a final volume of 100 μl, then [³⁵S]CPP32 (4 μl) was combined with concentrated fraction (10 μl) and incubated at 37 °C for 4 h. Samples were resolved on 12% SDS-polyacrylamide gels and visualized by autoradiography.

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Beginning with lysate from the cytotoxic T-cell line MTL 2.8.2, which expresses high levels of granzyme B, we used fast protein liquid chromatography (FPLC; Pharmacia) to separate granzyme A (showing BLT esterase activity) and granzyme B (showing Asp-ase activity). The relevant fractions showed little, if any, contamination by each other or any other granzymes (Fig. 1a). Granzyme B cleaved *in vitro* translated CPP32, but granzyme A could not (Fig. 1b). The size of the band generated, ~20K, corresponds to cleavage between the p17 and p12 subunits of CPP32. This cleavage would occur at the sequence IETD-S, which conforms with the proposed substrate specificity of granzyme B based on molecular modelling (Asp at P₁ and a second acidic residue at P₃ (ref. 8)). Although a band does become visible following treatment of CPP32 by granzyme A, we believe this is due to cleavage at an inappropriate site because it is not the correct size to produce active enzyme. Incubation of CPP32 with whole MTL lysate resulted in disappearance of full-length CPP32 and a band at ~20K. In other experiments this band was not always present, owing to extensive degradation in the presence of multiple granzymes. In contrast, incubation with granzyme B always gave the p17 cleavage product.

To establish that cleavage of CPP32 was dependent on granzyme B proteolytic activity, we used a heterologous expression system¹⁹. Enzymatically active murine granzyme B, as well as the inactive zymogen and an active site Ser-Ala mutant (S183A)¹⁰, were expressed in COS cells, and the ability of lysates of these cells to cleave CPP32 was assessed. COS cell lysates that expressed active granzyme B cleaved CPP32, but those that produced inactive zymogen or the active site mutant did not (Fig. 2). The band seen with these lysates was the same size as that generated when CPP32 was incubated with granzyme B from MTL (Fig. 1b).

Similar lysates were used to prove that the cleavage mediated by granzyme B produced active, PARP-cleaving, CPP32. Incubation of *in vitro* translated PARP with COS cell lysates expressing active granzyme B resulted in PARP cleavage to produce the 89K and 24K fragments found in apoptotic cells²⁰ (Fig. 3a), indicating that CPP32 is indeed active in these cells. In

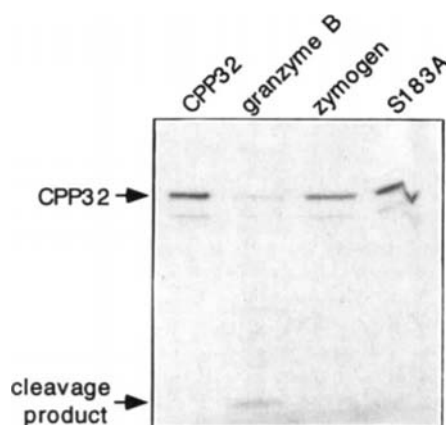


FIG. 2 Cleavage of CPP32 precursor by granzyme B-expressing COS lysates. [³⁵S]CPP32 precursor was combined with cytosolic lysates from COS cells expressing active granzyme B, the inactive zymogen of granzyme B or an active site Ser-Ala mutant of granzyme B (S183A). [³⁵S]CPP32 alone was used as a negative control.

METHODS. COS cell lysates expressing recombinant active granzyme B, the inactive zymogen, or the active site Ser-Ala mutant (S183A) were generated, and checked for Asp-ase activity, as described previously^{9,10,17}. [³⁵S]CPP32 (3 µl), generated as described above, was incubated with 50 µg total protein from COS cell lysates in 1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl at 37 °C for 1 h. Samples were separated on 12% SDS-polyacrylamide gels followed by autoradiography to detect cleavage products.

contrast, lysates containing the inactive zymogen form of granzyme B, or the active site Ser-Ala mutant, did not result in cleavage of PARP. An inhibitor study, shown in Fig. 3b, revealed that the PARP-cleaving activity was sensitive to the cysteine protease inhibitors iodoacetamide and *N*-ethylmaleimide, but not to E-64 (another cysteine protease inhibitor), and not to any serine protease inhibitors, an inhibitor profile characteristic of the ICE/*ced-3* family of proteases¹³. The activity of these lysates is related to CPP32 because Ac-DEVD-CHO¹³, a potent, specific inhibitor of CPP32, was able to inhibit the PARP cleavage of these COS cell lysates (Fig. 3b). Presence of active CPP32 in these COS cell lysates was confirmed using western blot analysis with an antibody directed against the p17 subunit of CPP32 (Fig. 4a). Again, active CPP32 was found only in lysates from COS cells expressing active granzyme B. Taken together, these results indicate that expression of granzyme B in COS cells results in activation of their endogenous CPP32, which is able to cleave PARP. Finally, cleavage of CPP32 was found in susceptible target cells following attack by CTLs (Fig. 4b). A role for PARP, and therefore CPP32, in CTL-mediated killing *in*

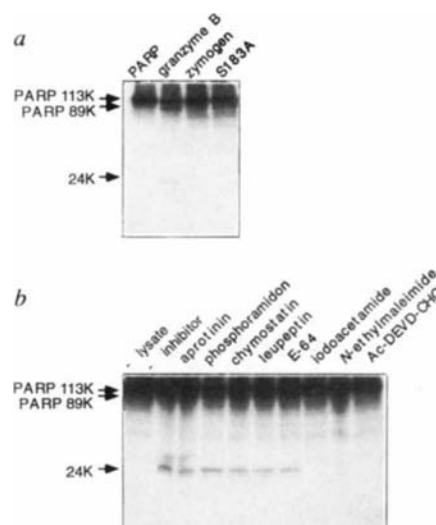


FIG. 3 PARP cleaving activity of granzyme B-expressing COS cell lysates. *a*, Cleavage of PARP by lysates expressing active recombinant murine granzyme B. [³⁵S]PARP was combined with cytosolic lysates from COS cells expressing active granzyme B, the inactive zymogen of granzyme B or an active site Ser-Ala mutant of granzyme B (S183A). [³⁵S]PARP alone was used as a negative control. *b*, Inhibition of PARP-cleaving activity of COS cell lysates. Cytosolic lysates from COS cells expressing active granzyme B were incubated with [³⁵S]PARP in the presence of the protease inhibitors indicated, or the synthetic tetrapeptide aldehyde inhibitor Ac-DEVD-CHO. Control samples were incubated either in the absence of COS cell lysate or in the presence of lysate but in the absence of inhibitor.

METHODS. *a*, COS cell lysates expressing recombinant active granzyme B, the inactive zymogen, or the active site Ser-Ala mutant (S183A) were generated, and checked for Asp-ase activity, as described previously^{9,10,17}. [³⁵S]PARP was generated using an *in vitro* transcription/translation kit as described¹². [³⁵S]PARP (3 µl) was incubated with 50 µg total protein from COS cell lysates in 1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl at 37 °C for 1 h. Samples were resolved by 14% SDS-polyacrylamide gels and cleavage products were visualized by fluorography. *b*, PARP cleavage by COS cell lysates expressing active granzyme B was assayed as for *a*, except that lysates were preincubated at 37 °C for 20 min with indicated protease inhibitors or the tetrapeptide aldehyde inhibitor. Incubation mixtures contained aprotinin (2 µg ml⁻¹), phosphoramidon (8.5 µM), chymostatin (100 µM), leupeptin (100 µM), E-64 (10 µM), iodoacetamide (5 mM), *N*-ethylmaleimide (5 mM), or Ac-DEVD-CHO (100 nM).

in vivo has previously been suggested by inhibition of CTL-mediated lysis by nicotinamide, a PARP inhibitor²¹.

In conclusion, we have shown that the CTL-specific serine protease granzyme B can cleave and activate the ICE-like protease CPP32, resulting in PARP cleavage. Production of the two fragments of PARP could lead to inhibition of DNA repair, activation of an endonuclease, depletion of NAD and ATP, and ultimately cell death. However, the role of PARP is controversial¹⁶⁻¹⁸. PARP is probably not the sole substrate of CPP32, but its cleavage acts as a marker for apoptosis and CPP32 activity. Additional substrates are likely to be found which are also critical to the induction of apoptosis. This demonstration of a physiological substrate for a granzyme that has been implicated in programmed cell death suggests that the two pathways to apoptosis (CTL-mediated and developmental cell death) coincide at this central point. Identification of an intracellular substrate for granzyme B as a protease which is part of an enzymatic cascade indicates that there may be numerous sites at

which cell-mediated immunity can be inhibited within the target cell. This information is crucial to the design and development of new immunosuppressants. □

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FIG. 4 Cleavage of CPP32 *in vivo*. *a*, Cleavage of CPP32 in COS cells expressing recombinant murine granzyme B, the inactive zymogen of granzyme B or an active-site Ser-Ala mutant of granzyme B (S183A). *b*, *In vivo* cleavage of CPP32 following CTL attack. Cleavage of CPP32 in target cells (EL4 or YAC-1) occurs following a 4-h incubation of these cells with a CTL clone (4 h), but not at time zero.

METHODS. *a*, COS cell lysates expressing recombinant active granzyme B, the inactive zymogen, or the active site Ser-Ala mutant (S183A) were generated, and checked for Asp-ase activity, as described previously^{9,10,17}. Total cell protein (50 µg) was incubated at 37 °C for 1 h and then resolved by 12% SDS/PAGE followed by blotting onto PVDF. CPP32 was detected using an antibody directed towards the p17 subunit of CPP32 followed by chemiluminescence. *b*, CTL21.9 (which lyse cells exclusively by the granzyme/perforin pathway²³), EL4 and YAC-1 cells were cultured as described previously²³. CTLs were combined with the target cells in a 1:1 effector-to-target ratio. At both time zero and following incubation at 37 °C for 4 h, CTLs were removed from the sample using Dynabeads (Dynal) according to the manufacturer's instructions. Once isolated, target cells were washed with PBS and then lysed in 10 mM HEPES/KOH, pH 7.4, 2 mM EDTA, 0.1% (w/v) CHAPS, 5 mM DTT. Total cell protein (50 µg) from the target cells was resolved and CPP32 was detected as described above.

Highly processive microtubule-stimulated ATP hydrolysis by dimeric kinesin head domains

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STUDIES of immobilized kinesin have shown that a single dimeric molecule can maintain contact with and drive sliding of a microtubule^{1,2}. In solution, however, native kinesin binds microtubules too weakly³ and hydrolyses ATP too slowly to produce the high sliding velocities seen in motility assays⁴. This apparent inhibition in solution appears to be caused by the binding of kinesin's tail domains to its motor (head) domains in a folded conformation⁵. DKH392, a construct containing two heads but no tails, has been shown to display both tight binding to microtubules and high ATPase rates⁶. Furthermore, it retains one molecule of ADP per dimer when bound to microtubules⁷, which could facilitate a 'hand-over-hand' mechanism for processive motion. Here we show that DKH392 hydrolyses more than 100 ATP molecules per diffusional encounter with a microtubule, even in the high-salt conditions encountered physiologically. This provides direct evidence that kinesin's activity is highly processive, with the motor remaining attached to a microtubule through many cycles of ATP hydrolysis.

The rate-limiting step for kinesin at subsaturating microtubule (MT) levels is MT-stimulated ADP release⁸⁻¹⁰, with $K_{0.5}^{MT}$ equal to the concentration of tubulin dimers that produce half-maxi-

mal ATPase activity (Fig. 1). DKH392 at moderate ionic strength⁶ has an apparent bimolecular rate, $k_{\text{cat}}/K_{0.5}^{\text{MT}}$, of $\geq 1000 \mu\text{M}^{-1} \text{s}^{-1}$, which defines the rate of bimolecular association of DKH392-ADP with the tubulin dimers of a MT if only one ATP molecule is hydrolysed per encounter. Simple diffusional association of spheres is fast in solution, but approach to the individual tubulin subunits of a MT is severely hindered, and the theoretical limit for bimolecular association of the DKH392-ADP complex with the individual tubulin subunits of a MT 1–10 μm in length is only $\sim 30 \mu\text{M}^{-1} \text{s}^{-1}$, as discussed previously¹¹. Thus $k_{\text{cat}}/K_{0.5}^{\text{MT}}$ is much larger than the theoretical limit ($\sim 30 \mu\text{M}^{-1} \text{s}^{-1}$), which suggests that DKH392 is processive with hydrolysis of many ATP molecules per encounter with a MT.

Net binding of kinesin to MTs is weaker, however, at higher ionic strength, and the relevance of processivity to the physiological situation and to motility assays in higher salt concentrations has been unclear. Figures 2 and 3 present parallel evaluation of both $k_{\text{cat}}/K_{0.5}^{\text{MT}}$ and K_{bi} in 120 mM potassium acetate in A25 buffer at a total ionic strength of 160 mM, where k_{bi} is the bimolecular rate for productive encounter of DKH392-ADP with tubulin dimers leading to release of ADP (Fig. 1). The concentration of DKH392 was 10 nM (heads) which is sufficiently low for interference between motors on the MT lattice to be negligible under these conditions. The k_{cat} and $K_{0.5}^{\text{MT}}$ values are 38s^{-1} (per head) and 240 nM, respectively (Fig. 2a, b). To determine the k_{bi} value, experiments were performed in which DKH392 was preloaded with [³²P]ADP, mixed with MTs, and the release of [³²P]ADP followed using a cold chase

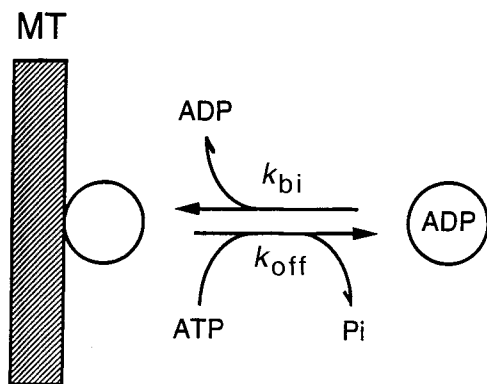
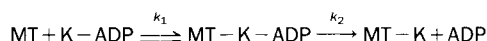


FIG. 1 Minimal scheme for MT-stimulated ATP hydrolysis by kinesin. At subsaturating concentrations of MTs, the rate limiting step is MT-stimulated ADP release, by k_{bi} , leading to formation of a tightly bound rigor-like complex of a MT and head without bound nucleotide⁷. This net reaction is expected to consist of at least two steps with initial binding of the kinesin-ADP complex to the MT followed by release of ADP:



where K is kinesin. For this scheme, k_{bi} is equal to $k_1 k_2 / (k_2 + k_{-1})$. If $k_{-1} \geq k_2$ then some diffusional encounters of K-ADP with a MT will not be productive (result in ADP release) and k_{bi} will be less than the actual diffusional encounter rate, k_1 . Dimeric head domains bind through only one head with the other tethered head retaining bound ADP⁷. The pseudo-first-order rate constant for net (multistep) ATP-induced release of the head domain resulting in diffusional separation of the head from the MT is k_{off} . If only one ATP is hydrolysed for each cycle of net binding and release of kinesin from the MT, then k_{off} at saturating ATP is equal to the k_{cat} value and $k_{\text{cat}}/K_{0.5}^{\text{MT}} = k_{\text{bi}}$. Processivity would short-circuit this scheme and allow multiple ATP molecules to be hydrolysed during each productive diffusional encounter. The tethered nature of the complex of dimeric kinesin with a MT provides a reasonable mechanism for generation of extensive processivity through alternating interaction of the two heads without net diffusional release of the dimer⁷.

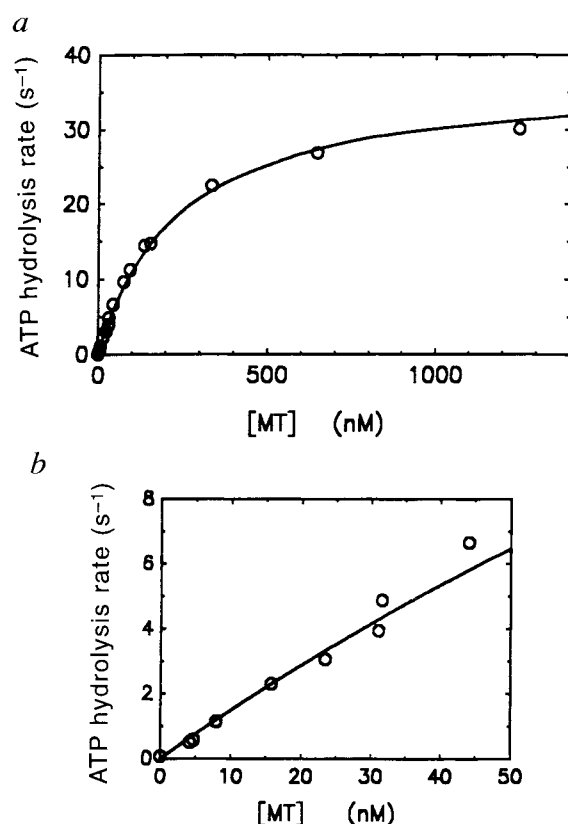


FIG. 2 Kinetics of MT-stimulated ATP hydrolysis of DKH392 in 120 mM K-acetate. a, MT-stimulated ATP hydrolysis of DKH392. The curve is a theoretical plot for a k_{cat} of 38s^{-1} and a $K_{0.5}^{\text{MT}}$ of 240 nM. b, Expanded plot at low concentration of MTs.

METHODS. DKH392 and MTs were prepared as described previously^{4,15}. ATPase was determined at 25 °C in A25 buffer (25 mM potassium *N*-(2-acetamido)-2-aminoethanesulphonic acid, pH 6.9, 2 mM magnesium acetate, 2 mM potassium EGTA, 0.01 mM potassium EDTA, 1 mM 2-mercaptoethanol) supplemented with 120 mM potassium acetate, 1 mM Mg-ATP, 2 mM potassium phospho(enol)pyruvate, 0.3 mM NADH, $15 \mu\text{g ml}^{-1}$ pyruvate kinase, $5 \mu\text{g ml}^{-1}$ lactate dehydrogenase and 3 μM taxol as described previously⁴.

of excess ATP and pyruvate kinase as a trap for the released ADP (see ref. 9). At all concentrations of MTs, ADP release obeys first-order kinetics with no indication of differentiation between the head domains of dimeric DKH392 (Fig. 3a). The rate increases linearly with the concentration of MTs and has a bimolecular rate constant, k_{bi} , of $2.7 \mu\text{M}^{-1} \text{s}^{-1}$ (Fig. 3b). Thus the steady state $k_{\text{cat}}/K_{0.5}^{\text{MT}}$ value under these higher salt conditions is still 60-fold larger than k_{bi} , and 120 ATP molecules are hydrolysed during each productive encounter of dimeric DKH392 with a MT. 80PM is a buffer of high ionic strength that is often used for single motor motility assays^{1,2}. The kinetic parameters of DKH392 in 80PM were also determined and are similar (44s^{-1} and 270 nM for k_{cat} and $K_{0.5}^{\text{MT}}$, respectively). The k_{bi} value was determined in both buffers by MT-stimulated release of fluorescent 2'(3')-*O*-(*N*-methylanthraniloyl)-adenosine 5'-diphosphate (mant-ADP) in the presence of excess ATP, and values of 2.4 and $2.7 \mu\text{M}^{-1} \text{s}^{-1}$ were obtained for A25 with 120 mM potassium-acetate and 80PM, respectively. The similarity of the values for k_{bi} determined in 120 potassium-acetate using either [³²P]ADP or mant-ADP is consistent with other observations (refs 10, 12) and J. Nagy and D.D.H., unpublished observations) and indicates that the k_{bi} value for mant-ADP release in 80PM is likely to be equivalent to the ADP release rate. DKH405 is also dimeric¹³ and has similar k_{cat} and $K_{0.5}^{\text{MT}}$

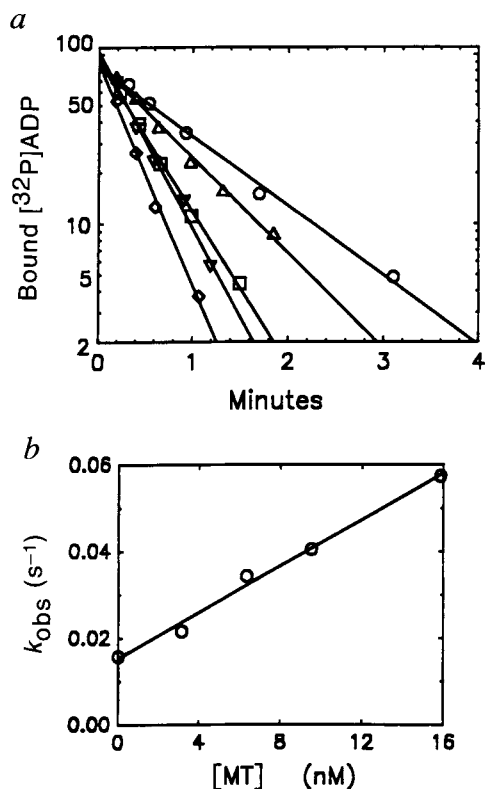


FIG. 3 Kinetics of MT-stimulated release of ADP from DKH392-ADP in 120 mM K-acetate. *a*, Loss of $[^{32}\text{P}]\text{ADP}$ following dilution of DKH392- $[^{32}\text{P}]\text{ADP}$ into a cold ATP chase. MTs were present in the chase at 0 (circles), 3.2 (triangles), 6.3 (squares), 9.5 (inverted triangles) and 15.8 nM (diamonds). *b*, Dependence of the observed first-order rate for ADP release on the MT concentration. Data were fitted to a straight line and the slope of $2.7 \mu\text{M}^{-1} \text{s}^{-1}$ equals the second-order rate constant, k_{bi} .

METHODS. Reaction conditions were as Fig. 2 except that lactate dehydrogenase and NADH were omitted. DKH392 at 20 μM was incubated with 5 μM $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ for 20 min at 25 $^{\circ}\text{C}$ to equilibrate the bound ADP with $[^{32}\text{P}]$. Pyruvate kinase and phospho(enol)pyruvate were included in the equilibration so that kinesin would be undergoing steady-state ATP hydrolysis. The DKH392- $[^{32}\text{P}]\text{ADP}$ complex was then diluted (10 nM final) into the reaction mixture supplemented with 1 mM Mg-ATP and the loss of pyruvate kinase-inaccessible $[^{32}\text{P}]\text{ADP}$ was determined following an acid quench as described previously⁹. In this reaction the $[^{32}\text{P}]\text{ADP}$ that is tightly bound to DKH392 is inaccessible to pyruvate kinase and remains as $[^{32}\text{P}]\text{ADP}$, whereas the $[^{32}\text{P}]\text{ADP}$ that is released is converted to $[^{32}\text{P}]\text{ATP}$. Because there is excess unlabelled ATP present, this free $[^{32}\text{P}]\text{ATP}$ will not rebinding to DKH392, and thus the rate of loss of $[^{32}\text{P}]\text{ADP}$ equals the rate at which it is released from the DKH392-ADP complex.

values of 46s^{-1} and 320 nM, respectively, in 120 mM potassium acetate.

Because physical binding of DKH392 to MTs parallels activation of the ATPase reaction⁶, the rate constant for net diffusional escape of kinesin from a MT at saturating ATP, k_{off} , can be determined (see Fig. 1) from the net binding rate, k_{bi} , and $K_{0.5}^{\text{MT}}$ as: $K_{0.5}^{\text{MT}} = k_{\text{off}}/k_{\text{bi}}$, and $k_{\text{off}} = k_{\text{bi}} K_{0.5}^{\text{MT}}$. Using the data in 80PM, $k_{\text{off}} = 2.7 \mu\text{M}^{-1} \text{s}^{-1} \times 0.27 \mu\text{M} = 0.73 \text{s}^{-1}$, and the average persistence time before release from the MT is 1.4 seconds. For a step size of 8nm^{14} per ATP, the sliding velocity will be $44 \text{s}^{-1} \times 2 \text{heads/dimer} \times 8 \text{nm} = 0.7 \mu\text{m s}^{-1}$, and the average run length before release will be $\sim 1 \mu\text{m}$. This calculation is in good agreement with previous motility estimates made by Block *et al.*², in 80PM for beads containing a single kinesin, for which the observed average distance for a bead to travel before release from the MT was $1.4 \mu\text{m}$ at a velocity of $350\text{--}450 \text{nm s}^{-1}$. Thus most of their observed processive movement of beads containing a single kinesin can be accounted for by the inherent processivity of dimeric kinesin itself. The value of $1.4 \mu\text{m}$ includes some events in which kinesin would have diffusionally escaped from the MT, but recapture occurred before the slow diffusion of the bead could remove it from the vicinity of the MT. An even greater contribution is likely for sliding of MTs over single kinesin attached to glass², and results in the longer run lengths observed by Howard *et al.*¹. Block *et al.*² observed that application of an optical trap behind a moving bead caused shorter run lengths of $0.8 \mu\text{m}$. Because the trap would rapidly separate a

bead from its position on the MT if the kinesin detached, this length is a more appropriate measure of the run lengths of isolated kinesin, and agrees well with the above estimate for free kinesin determined from its MT-stimulated ATPase kinetics in solution. Most of the tubulin subunits in the preparations used here are in MTs of 3 μm or greater. This is only slightly longer than the average run length, and the observed processivity with DKH392 might be limited at least in part by the length of the MTs.

Although Gilbert *et al.*¹² presented evidence for a low level of processivity with a longer head construct, K401, under some circumstances, their value of $k_{\text{cat}}/K_{0.5}^{\text{MT}}$ was equal to their observed k_{bi} , which is incompatible with hydrolysis of more than one ATP per head by K401 per diffusional encounter with a MT. The basis for this discrepancy with the DKH392 results is unclear but could involve the average MT length and other experimental conditions. The difference is not likely to be due to the increased length of K401 as opposed to DKH392 because the longer DKH405 is similar to DKH392.

DKH346 and DKH357 are monomers¹³ and they have ratios of $k_{\text{cat}}/K_{0.5}^{\text{MT}}$ to k_{bi} that are small compared to DKH392, but may be greater than one (J. Nagy, W. Jiang and D.D.H., unpublished observations). This is consistent with either limited rebinding of released monomer heads to the same MT or failure to release during every ATPase cycle, but is clearly differentiated from the extensive processivity exhibited by dimeric DKH392. □

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Polarity-specific activities of retinoic acid receptors determined by a co-repressor

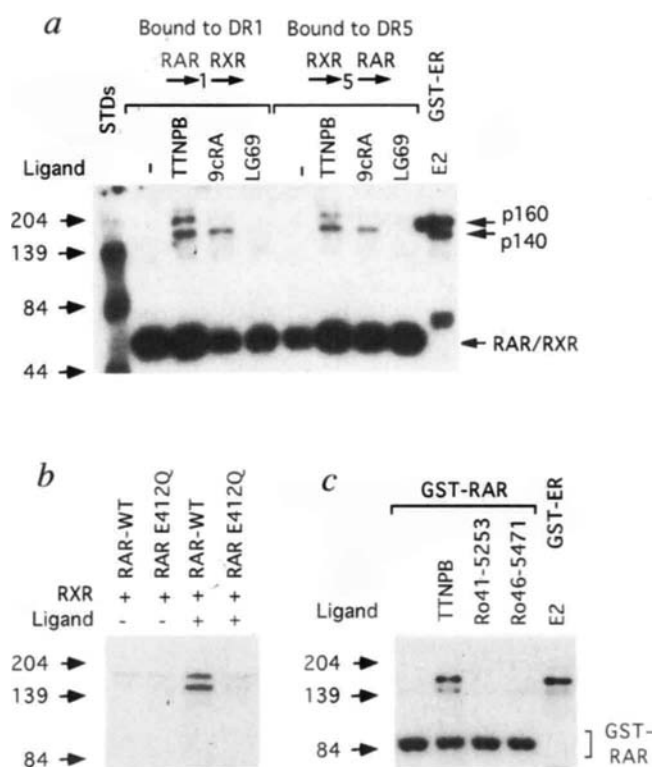
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RETINOIC acid receptors (RARs) and retinoid-X receptors (RXRs) activate or repress transcription by binding as heterodimers to DNA-response elements that generally consist of two direct repeat half-sites of consensus sequence AGGTCA (reviewed in ref. 1). On response elements consisting of direct repeats spaced by five base pairs (DR + 5 elements), RAR/RXR heterodimers activate transcription in response to RAR-specific ligands, such as all-*trans*-retinoic acid (RA)². In contrast, on elements consisting of direct repeats spaced by one base pair (DR + 1 elements), RAR/RXR heterodimers exhibit little or no response to activating ligands and repress RXR-dependent transcription³. Here we show that ligand-dependent transactivation by RAR on DR + 5 elements requires the dissociation of a new nuclear receptor co-repressor, N-CoR, and recruitment of the putative co-activators p140 and p160 (refs 4, 5). Surprisingly, on DR + 1 elements, N-CoR remains associated with RAR/RXR heterodimers even in the presence of RAR ligands, resulting in constitutive repression. These observations indicate that DNA-response elements can allosterically regulate RAR-co-repressor interactions to determine positive or negative regulation of gene expression.

The differential response of RAR to activating ligands on DR + 1 and DR + 5 response elements result from opposite polarities of heterodimer binding to the asymmetrically oriented half-sites⁶. On DR + 5 sites, RAR/RXR heterodimers bind such that RAR occupies the downstream half-site^{7,9}, whereas on DR + 1 elements, RAR occupies the upstream half-site⁶. Mutations in the RAR and RXR that reverse these polarities of binding also reverse the pattern of transcriptional responses to RAR-specific ligands⁶. These observations suggested the existence of co-activator or co-repressor proteins that could distinguish polarity-specific conformations of RAR/RXR heterodimers bound to DR + 1 and DR + 5 elements. To search for such proteins, a DNA-dependent assay for protein-protein interactions was developed (Fig. 1). Using CV1 whole-cell extracts, two proteins of approximate relative molecular mass (M_r) 140K and 160K (p140 and p160, respectively) were observed to interact with RAR/RXR heterodimers bound to DNA only in the presence of RAR ligands, such as the RAR-specific ligand TTNPB, or the pan-agonist 9-*cis*-RA (Fig. 1a). The interactions of p140 and p160 were selective for the RAR component of the heterodimer because the RXR-specific ligand,

FIG. 1 Proteins of apparent M_r 140K and 160K interact with RAR/RXR heterodimers bound to DR + 1 and DR + 5 elements in a ligand-dependent manner. Purified human RAR- α and human RXR- α were bound to double-stranded, biotinylated oligonucleotides containing either a DR + 1 or DR + 5 recognition motif, captured on streptavidin agarose and incubated with whole-cell extracts in the presence or absence of 10^{-6} M concentrations of the indicated ligands. After extensive washing, proteins interacting with RAR/RXR heterodimers were recovered from the streptavidin agarose by boiling in SDS sample buffer, resolved by electrophoresis on SDS polyacrylamide gels and transferred to nitrocellulose membranes. Proteins capable of forming direct interactions with RAR in the presence of ligand were detected using liganded ³²P-GST-RAR. a, RAR-specific (TTNPB), but not RXR-specific (LG69), ligands promote the interaction of 140K and 160K proteins with RAR/RXR heterodimers bound to DR + 1 and DR + 5 elements. The pan-agonist 9-*cis*-RA preferentially promotes the interaction of the 140K protein. The strong signal at 54K represents dimerization of the ³²P-GST-RAR probe with RXR and RAR recovered from the streptavidin-agarose matrix, providing a measure of the extent of heterodimer initially bound to the biotinylated response elements. b, Interaction of p140 and p160 with RAR is dependent on the RAR AF-2 domain. RAR E412Q contains a mutation in the AF-2 region that inhibits transcriptional activation but does not affect ligand binding¹⁴. c, Interaction of p140 and p160 is induced by RAR agonists but not antagonists. Ro46-5471 and Ro41-5253 are antagonists that block RA-dependent transcription and compete for the binding of all trans RA to RAR²⁰. METHODS. Oligonucleotides were synthesized to contain biotin residues at the 5' end using a biotin-phosphoramidite (Applied Biosystems). The sequence of the sense strand of the DR + 5 element was: 5'-B-AAGGGG-ATCCGGGTAGGGTTACCGAAAGTTCACGAGATCT-3'. The sequence of the sense strand of the DR + 1 element was 5'-B-AAGGGGATCCGTAC-AGGTCACAGGTCACGAGATCT-3'. Purified, double-stranded oligonucleotide (1 μ g) was incubated with 100 ng each of purified RAR and RXR produced as GST fusion proteins in *Escherichia coli* and cleaved from the GST portion using thrombin. The DNA binding buffer consisted of 8 mM Tris-phosphate, pH 7.4, 0.12 M KCl, 8% glycerol, 4 mM DTT, 0.5% CHAPS (Boehringer Mannheim). Receptor-DNA complexes were captured using 25 μ l of a slurry of streptavidin agarose and washed five times with the washing buffer H (20 mM HEPES, pH 7.7, 50 mM KCl, 20% glycerol, 0.1% NP-40). Receptors were then incubated in the binding buffer with the indicated ligands or solvent alone for 30 min at room temperature. CV1 whole-cell extract (1.5 mg) (prepared as previously described⁴) was then added to the receptor-DNA complexes and incubated at 4 °C for 1 h. Complexes were washed three times with the washing buffer H, resolved by electrophoresis on SDS polyacrylamide gels and transferred to nitrocellulose membranes. To prepare an RAR probe, a complementary DNA encoding DNA and LBDs of human RAR- α was inserted in frame in the pGEX-2TK vector to introduce a phosphorylation site for cardiac kinase. The resulting GST-RAR fusion protein was labelled with ³²P and used to detect proteins bound to nitrocellulose filters in the presence of 10^{-6} M all-*trans*-RA as previously described²¹.



LG69, failed to induce an interaction with either protein, consistent with the previously reported inhibitory effects of RAR on the binding of ligands to RXR^{6,10}. Although other factors that interact with RAR may serve critical functions^{11,12}, several lines of evidence suggest that p140 and p160 are involved in ligand-dependent transactivation. First, interactions with p140 and p160 depended on the AF-2 region of the RAR ligand-binding domain. This region is conserved among many members of the nuclear receptor family¹³⁻¹⁶ and is required for ligand-dependent transcription (Fig. 1b). Second, RAR antagonists that compete for the binding of all *trans*-RA to the RAR did not induce the binding of p140 and p160 to RAR, indicating a requirement for activating ligands (Fig. 1c). Third, p140 and p160 appear to be equivalent to previously described co-activator proteins that interact with the ligand-binding domain (LBD) of the oestrogen receptor (ER)^{4,5}, as ³²P-RAR was capable of detecting these proteins bound to the glutathione *S*-transferase (GST)-ER-LBD (Fig. 1a, lane 10). However, p140 and p160 interacting equivalently with RAR on the DR + 1 and DR + 5 elements (Fig. 1a) raised the possibility that altered co-activator binding did not account for the differential activities of RAR on the two elements.

We therefore next investigated the possibility that co-repressors^{17,18} might interact differentially with RAR/RXR heterodimers in a DNA-site-specific fashion in the absence of ligand, using unliganded ³²P-GST-RAR as a probe. A protein of approximately 270K (p270) was observed to interact with RAR/RXR heterodimers in the absence of ligand on both the DR + 1 and DR + 5 elements (Fig. 2a). Remarkably, TTNPB and 9-*cis*-RA prevented the interaction of p270 with heterodimers bound to the DR + 5 element, but not with heterodimers bound to the DR + 1 element. The RXR-specific ligand, LG69, had no effect on the binding of this protein to RXR/RAR heterodimers on either response element. The interaction of p270 with RAR/RXR heterodimers was thus regulated both by the RAR ligand and the polarity of its binding to DNA, consistent

with the possibility that it could serve as a polarity-specific co-repressor.

To determine whether p270 interacted with RAR but not RXR, experiments were performed using individual receptors presented as GST fusion proteins in the absence of DNA. Although GST-RAR interacted strongly with p270, little or no interaction was observed with GST-RXR¹⁹ (data not shown), indicating that the binding of p270 to RAR/RXR heterodimers is through RAR. Experiments performed with nuclear extracts derived from several different cell lines indicated that the 270K protein that interacts with RAR is broadly expressed and represents the major protein detected by this assay in all cells examined, although some cell types also contain minor bands in the size range 140K to 250K (Fig. 2b). These observations raised the possibility that p270 corresponded to N-CoR, a protein of similar *M_r* that was identified on the basis of a two-hybrid screen in yeast for proteins interacting with the unliganded thyroid-hormone receptor (TR)¹⁹. A polyclonal antiserum that was raised against recombinant murine N-CoR specifically interacted with p270 purified from mouse L-cell and P19-cell extracts using GST-RAR (Fig. 2b), indicating that p270 and N-CoR are the same protein.

To define the primary sequence determinants in RAR required for interaction with N-CoR, experiments were performed using GST-RAR deletion mutants. These experiments indicated that the hinge and part of the RAR LBD, but not the carboxy-terminal AF-2 region, were required for N-CoR interaction (Fig. 3a), as was the case for the TR¹⁹. The identification of the hinge region as being critical for interaction with N-CoR was of particular interest because this region is likely to undergo significant conformational changes dependent on the polarity of binding of RAR/RXR heterodimers to direct repeat elements. Rotation of the DNA binding domain with respect to the LBD must occur to allow RAR and RXR to switch between the upstream and downstream half-sites of direct repeat elements⁶⁻⁸. The resulting conformational differences in the hinge region might thus pro-

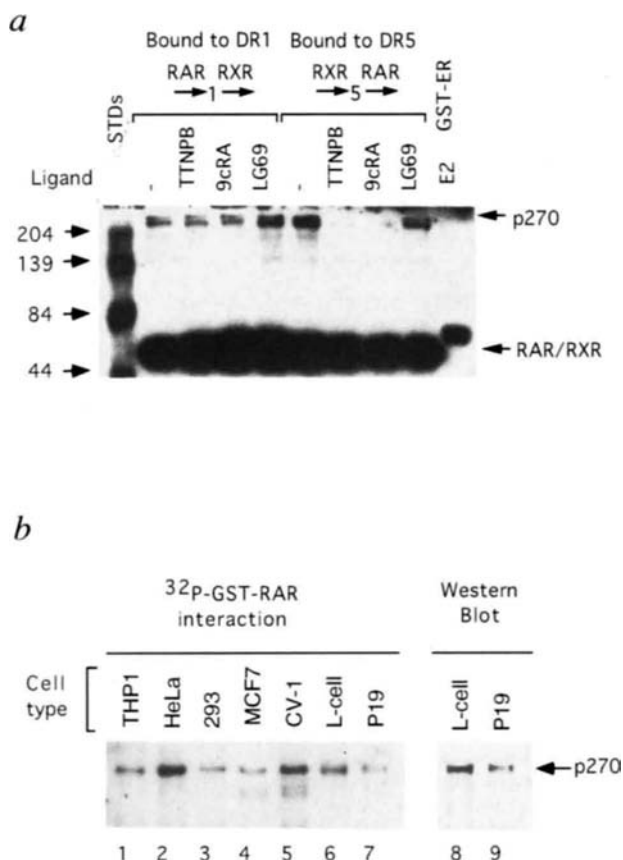


FIG. 2 The interaction of p270 (N-CoR) with RAR/RXR heterodimers is regulated by both RAR ligands and the polarity of heterodimer binding to direct repeat elements. **a**, Identification of a 270K protein that interacts differentially with heterodimers bound to DR + 1 and DR + 5 elements. Whole-cell extracts derived from CV1 cells were incubated with DNA-bound RAR/RXR heterodimers. After thorough washing, specifically bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Proteins capable of interacting with the RAR in the absence of ligand were detected using an unliganded ³²P-GST-RAR probe. This probe detected a 270K protein (p270/N-CoR) that interacted with unliganded RAR/RXR heterodimers bound to both DR + 1 and DR + 5 elements and was selectively released by RAR ligands on the DR + 5 element. **b**, p270 is broadly expressed and is recognized by an anti-N-CoR antiserum. Whole-cell extracts from the indicated cell types were incubated with an unliganded GST-RAR fusion protein bound to glutathione agarose. After washing, the specifically associated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Proteins in lanes 1–7 were detected using an unliganded ³²P-GST-RAR probe as described in **a**. Proteins in lanes 8 and 9 were detected using an anti-N-CoR antiserum.

METHODS. The methods for **a** were identical to those used for Fig. 1a, except that unliganded ³²P-GST-RAR was used as a probe. For **b**, GST-RAR was purified on glutathione-agarose and incubated with whole-cell extracts (1.5 mg cell protein) prepared from the indicated cell types. Receptor-associated proteins were then detected with ³²P-GST-RAR as described for **a**. For the western blot analysis of p270 (N-CoR) in **b**, the nitrocellulose membrane was incubated with guinea-pig polyclonal antisera raised against recombinant murine N-CoR at a 1:500 dilution.

vide the structural basis for the polarity-specific interactions of N-CoR with RAR. Alignment of the hinge region revealed a region of 50% amino-acid similarity between RAR- α and TR- β (Fig. 3b; see also ref. 19), but not other nuclear receptors. Because structural algorithms predict that this portion of the hinge region forms an α -helix, proline or glycine residues were introduced in place of conserved residues to break the putative helical structure and replace residues likely to be involved in protein-protein interactions. Several of these mutations, including L187P, A194P, 187-188GG and 194-195GG, severely weak-

ened the interaction of RAR with N-CoR (Fig. 3b), but had no effect on DNA or ligand binding (data not shown). We therefore refer to this region as the CoR box.

If N-CoR functions as a polarity-specific co-repressor, RARs containing mutations in the CoR-box that impair N-CoR interaction would be predicted to activate transcription on both DR+1 and DR+5 elements. To test this prediction, transient transfection experiments were performed using mutant and wild-type RARs in CV1 cells. The contributions of endogenous RARs to ligand-dependent transcription on DR+5 elements were elim-

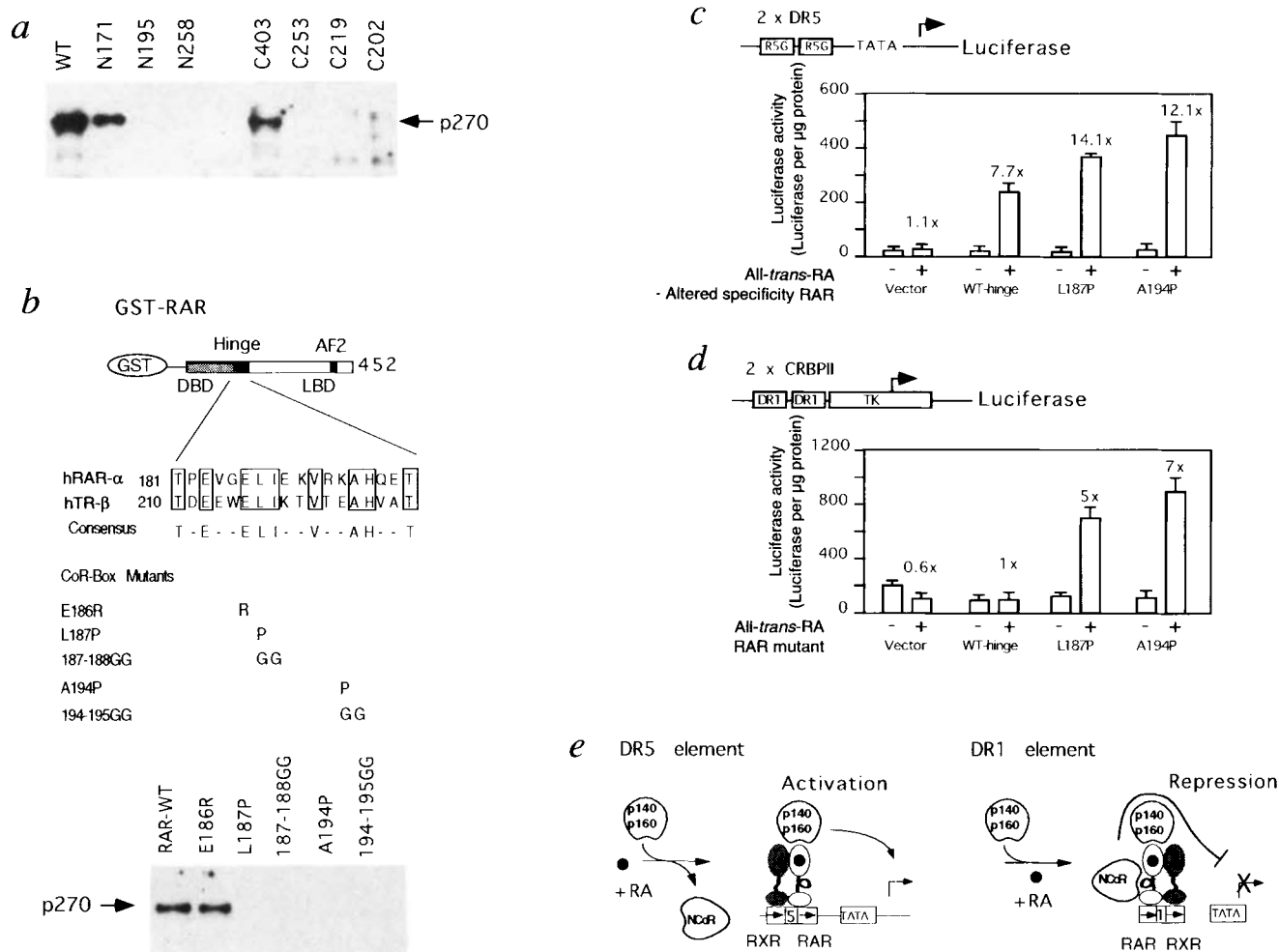


FIG. 3 N-CoR functions as a polarity-specific co-repressor. **a**, The hinge region of RAR- α is required for p270/N-CoR interaction. GST-RAR fusion proteins were expressed containing N-terminal deletions of RAR information at amino acids 171, 195 and 258, or C-terminal deletions at amino acids 403, 258, 219 and 202, and tested for their ability to interact with p270/N-CoR. Deletions of the hinge region (amino acids 171-195) and N-terminal region of the LBD abolished interaction. **b**, Point mutations in the hinge region of RAR- α that abolish interactions with N-CoR. Alignment of a conserved region within the hinge of hRAR- α and hTR- β is illustrated along with the positions of point mutations that were introduced into this region of RAR- α . Interactions of p270 (N-CoR) with GST-RAR fusion proteins containing these mutations are illustrated at the bottom. **c**, Mutations that impair interaction of N-CoR with the RAR do not impair ligand-dependent transactivation. Transcriptional activities of RARs containing wild-type and mutant hinge regions were tested on a promoter containing DR+5 in CV1 cells. To eliminate contributions of endogenous RARs, the DNA binding specificities of transfected RARs were changed to those of the glucocorticoid receptor. Together, the DR+5-containing response elements were modified to replace the downstream half-site with the glucocorticoid receptor half-site, AGAACA, resulting in the chimaeric DR+5 element, R5G. **d**, RARs

containing CoR box mutations activate transcription from a promoter containing DR+1 elements, but the wild-type RAR does not. **e**, Transcriptional activation in response to ligand requires both the dissociation of N-CoR and the recruitment of p140, p160 or other co-activators. Failure to activate transcription in response to ligand on a DR+1 site reflects the constitutive association of N-CoR, rather than differential recruitment of co-activators.

METHODS. Truncated cDNAs encoding the indicated deletions of the RAR were generated using polymerase chain reaction (PCR) and sub-cloned into the pGEX GST-based vector. Point mutations in the hinge region were generated using site-directed mutagenesis and confirmed by sequence analysis. Interaction assays were performed as described for Fig. 2b. The glucocorticoid P-box residues required for specific recognition of the AGAACA half-site were introduced into wild-type and CoR-box mutant RARs using site-directed mutagenesis. Transient transfection assays were performed using 10 ng of RSV-RAR expression vectors, 10 ng CMV-RXR expression vector and 100 ng of luciferase reporter plasmid in 1-cm dishes containing approximately 5×10^4 CV1 cells. The R5G and CRBP11 promoters and transfection conditions have been described previously⁶.

inated by changing the DNA binding specificities of the transfected receptors to those of the glucocorticoid receptor⁶. These altered-specificity RARs were then tested on promoters containing DR + 5 in which the downstream half-site was mutated to the glucocorticoid-receptor core recognition motif, AGAACA. The altered-specificity RARs containing CoR box mutations (L187P, A194P) were more active in response to 10^{-8} M all-*trans*-RA than the corresponding RARs containing a wild-type hinge region, indicating that interaction with N-CoR was not required for transactivation (Fig. 3c). Remarkably, mutant RARs lacking the ability to interact with N-CoR were capable

of activating transcription from a DR + 1-containing promoter, but the wild-type RAR was not (Fig. 3d). Together, these data indicate that the molecular explanation for repression by RAR on a DR + 1 element reflects the failure of ligand to cause the dissociation of the co-repressor, N-CoR, rather than altering co-activator association (Fig. 3e). These observations imply that interactions with N-CoR are dominant with respect to ligand-dependent transactivation, and show how positive or negative transcriptional effects of RAR/RXR heterodimers can be allosterically controlled by the DNA sequences to which they bind. □

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A transcriptional co-repressor that interacts with nuclear hormone receptors

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TRANSCRIPTIONAL silencing mediated by nuclear receptors is important in development, differentiation and oncogenesis^{1–3}. The mechanism underlying this effect is unknown but is one key to understanding the molecular basis of hormone action. Here we identify a receptor-interacting factor, SMRT, as a silencing mediator (co-repressor) for retinoid and thyroid-hormone receptors. SMRT is a previously undiscovered protein whose association with receptors both in solution and bound to DNA-response elements is destabilized by ligand. The interaction with mutant receptors correlates with their transcriptional silencing activities. *In vivo*, SMRT functions as a potent co-repressor, and a GAL4 DNA-binding domain fusion of SMRT behaves as a frank repressor of a GAL4-dependent reporter. Together, our results identify a new class of cofactors which may be important mediators of hormone action.

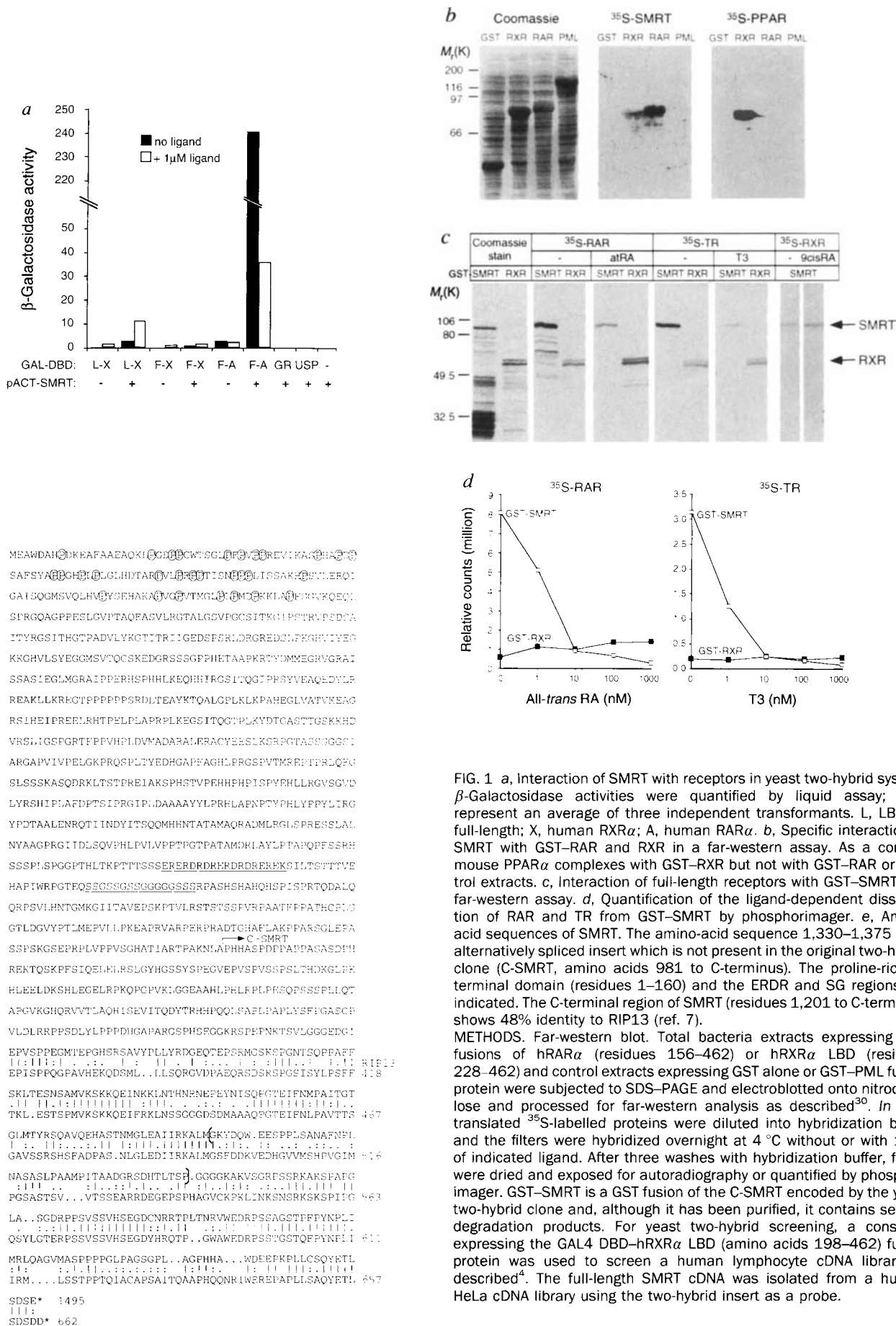
Using a GAL4 DNA-binding domain (DBD)–RXR fusion in a yeast two-hybrid screening⁴, we isolated several complementary DNA clones. One of these clones, SMRT, interacts strongly with unliganded full-length retinoic-acid receptor (RAR) but weakly or not at all with other receptors (Fig. 1a), and was selected for further characterization. In yeast, the ligand-binding domain (LBD) of the RAR and thyroid-hormone receptor (TR) is efficient in the interaction, whereas in both cases this interaction is reversed by ligand (data not shown). In a far-western analysis, SMRT preferentially complexes with immobilized RAR, marginally associates with the retinoid-X receptor (RXR) and shows no association with control polypeptides (Fig. 1b). In contrast, the peroxisomal proliferator-activated receptor (PPAR) selectively associates with RXR but not with RAR. Similarly, RAR or TR interacts strongly with immobilized

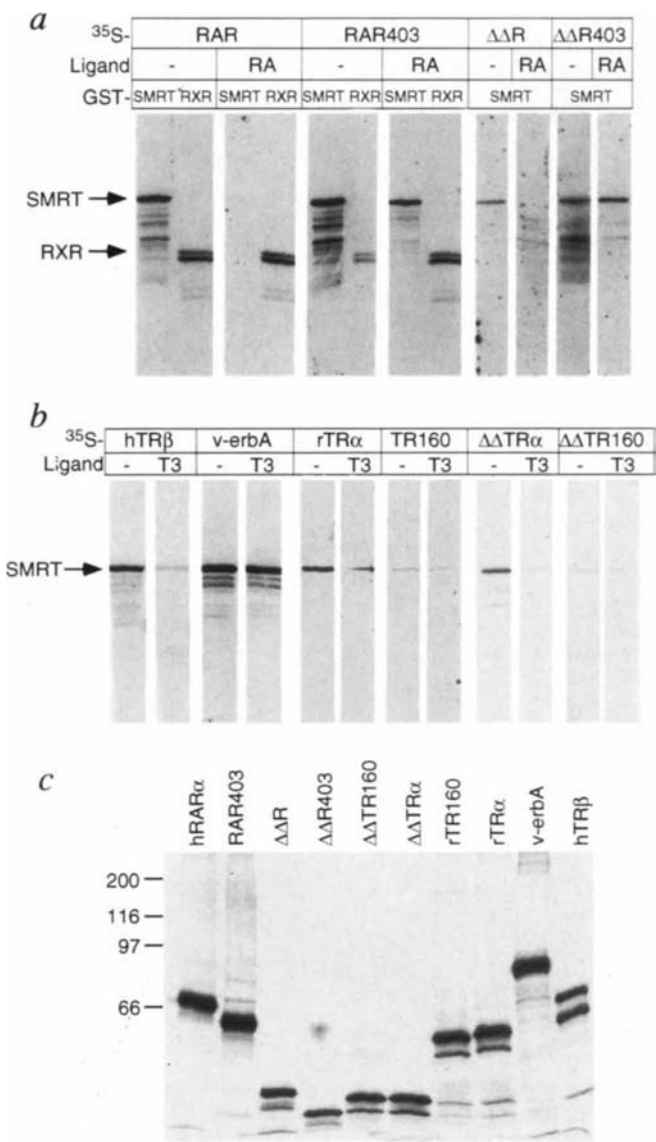
SMRT and RXR but not with glutathione-S-transferase (GST) or bacterial proteins, whereas RXR interacts weakly with SMRT (Fig. 1c). Binding of ligand to RAR or TR reduces their interactions with SMRT but not with RXR, and binding of ligand to RXR has slight effect (Fig. 1a, c). Figure 1d quantitates the dose-dependent release of SMRT from RAR or TR by ligand, which is very close to the dissociation constants^{5,6}.

Full-length SMRT encodes a polypeptide of 1,495 amino acids with an estimated relative molecular mass of 168,000 and is rich in proline and serine residues (Fig. 1e). GenBank comparison reveals similarity of the carboxy-terminal domain to a partial cDNA encoding another receptor-interacting protein, RIP13 (ref. 7) (Fig. 1e), whose role in receptor signalling is unknown. Northern blot analysis indicates that SMRT is ubiquitously expressed in low quantities and immunofluorescence data suggest that SMRT is a nuclear protein (data not shown).

Unlike other nuclear receptors, unliganded RAR and TR possess a strong silencing domain which represses basal-level promoter activity of their target genes^{8,9}. The preferential interaction of SMRT with RAR and TR in the absence of hormone suggests that SMRT may play a role in mediating the transcriptional silencing effect of receptors. To investigate further the involvement of SMRT in silencing, we tested its interactions with mutants that display distinct silencing and/or transactivation activities. Figure 2a shows that full-length and the LBD of RAR both associate with SMRT and that this association is blocked by ligand. The C-terminal-deletion mutant RAR403 is a potent suppressor of basal promoter activity of RAR target genes^{10,11}. As expected RAR403 and its amino-terminal-deletion derivative, $\Delta\Delta$ R403, interact strongly with SMRT in either the presence or absence of ligand, consistent with SMRT mediating the repressor activity of this mutant.

We next analysed the interactions of SMRT with two different classes of TR mutants: first, the oncogene *v-erbA*, which has strong silencing ability but no transactivation activity^{5,12,13}, and second, the Pro160→Arg point mutant *TR160*, which loses its capacity in basal-level suppression but retains hormone-dependent transactivation^{14,15}. Figure 2b shows that SMRT interacts strongly with *v-ErbA* both in presence or absence of ligand, but there is little interaction with *TR160* mutant. These data strongly support the idea that SMRT mediates transcriptional silencing





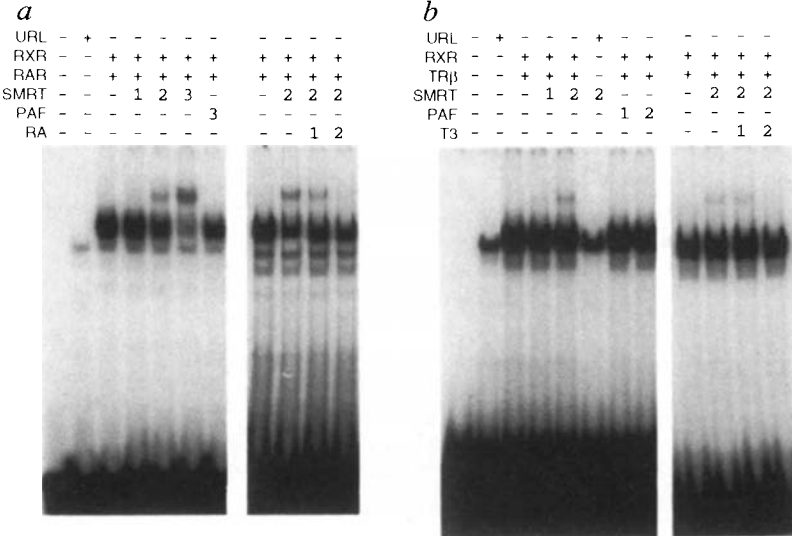
effects of these two receptors; the release of SMRT could be a prerequisite in ligand-dependent transactivation.

RAR and TR form heterodimers with RXR, resulting in a complex with high DNA-binding ability^{16,18}. We tested whether SMRT could interact with receptor-DNA complexes. Figure 3a shows that SMRT forms a ternary complex with the RXR-RAR heterodimer on a RAR-response element and that addition of ligand releases SMRT from this complex. Similarly, SMRT forms a ternary complex with the RXR-TR heterodimer on a TR-response element and addition of T3 disrupts the formation of this complex (Fig. 3b). These data show that SMRT may be recruited to target promoters by interaction with DNA-binding unliganded receptors in a ligand-reversible manner.

It has been shown that overexpression of RAR or TR can reverse the transcriptional silencing effect of the GAL4-DBD fusion of TR (GAL-TR) or RAR (GAL-RAR)^{19,20}, presumably by competition for a limiting amount of a co-repressor. A similar effect is shown in Fig. 4a and b where overexpression of v-ErbA or RAR403 reverses the silencing effect of GAL-RAR and GAL-TR on the basal activity of a luciferase reporter. Co-expression of the full-length or the C-terminal domain of SMRT (C-SMRT) titrates out the v-ErbA or RAR403 competitor activity and re-endsows GAL-RAR and GAL-TR with silencing activity. As a control, neither v-ErbA nor SMRT show any effect on the transactivation activity of GAL-VP16 (Fig. 4c) or on the basal activity of GAL-RXR (Fig. 4a). Furthermore, the full-length SMRT but not C-SMRT can further suppress two- to threefold the basal level activity of promoters containing either RAR to TR response elements (data not shown). Thus, we propose that SMRT can functionally replace the transcriptional co-repressor of RAR and TR *in vivo*.

FIG. 2 *a*, Interaction of wild-type hRARα and deletion mutant RAR403 with SMRT. ³⁵S-labelled receptors were used as probes to hybridize immobilized GST-SMRT in the presence (10 μM) or absence of all-trans retinoic acid. The total bacteria extract expressing GST-RXR was included as a control. When quantified by phosphorimager, RAR403 shows a 4-fold stronger interaction with SMRT than does wild-type RAR. *b*, Interaction of the oncogenic v-ErbA and rTRα R160 mutant (TR160) with GST-SMRT in a far-western assay. When quantified by phosphorimager, the v-ErbA shows an 18-fold stronger interaction with SMRT than hTRβ, and the TR160 mutant shows a 10-fold lower signal than the rTRα. *c*, Autoradiograph showing the ³⁵S-labelled receptors on SDS-PAGE. RAR403: hRARα amino acids (aa) 1-403; ΔΔR: hRARα aa 154-462; ΔΔR403: hRARα aa 157-403; ΔΔTR160: LBD of rTRα with Pro 160 to Arg mutation; ΔΔTRα: rTRα aa 122-410; TR160: full-length rTRα with Pro 160 to Arg mutation.

FIG. 3 Interaction of SMRT with receptor-DNA complex and the effect of ligands. *a*, Interaction of SMRT with RXR-RAR heterodimer on a DR5 element comprising AGGTCA direct repeat spaced by five nucleotides in a gel retardation assay. The amounts of GST fusion protein added in this experiment are (1), 6 ng; (2), 60 ng; and (3), 600 ng. PAF is a PML-associated protein isolated from another yeast two-hybrid screen (J.D.C. *et al.*, unpublished result). *b*, Interaction of SMRT with RXR-TR heterocomplex on a MLV TRE element. The amount of GST fusion protein added in this experiment are (1), 10 ng; (2) 100 ng. **METHODS.** Gel retardation assay: *in vitro* translated receptor or unprogrammed reticulocyte lysate (URL) was incubated with 1 μg poly(dIdC) on ice for 15 min in a total of 20 μl containing 75 mM KCl, 7.5% glycerol, 20 mM HEPES (pH 7.5), 2 mM DTT and 0.1% NP-40, with or without ligand as indicated (1, 10 nM; 2, 100 nM). A ³²P-labelled double-stranded oligonucleotide probe was added into the binding reaction (10,000 c.p.m. per reaction), and the reaction was further incubated for 20 min at room temperature. The protein-DNA complex was separated on a 5% native polyacrylamide gel at 150 volts.



If SMRT is the mediator of TR and RAR silencing, then direct recruitment of SMRT to a heterologous promoter should result in repression of the basal activity, as demonstrated before for the Ssn6-Tup1 co-repressor^{21,22}. We tested this idea by fusing SMRT to the GAL4 DBD and analysed its effect on the basal activity of the GAL4-dependent reporter. Figure 4d shows that both the full-length SMRT and its N-terminal domain can silence the basal promoter activity to a similar degree to the GAL-TR fusion, whereas the C-terminal receptor-interacting domain shows no silencing activity. Thus the silencing and receptor association domains appear to be functionally distinct. These

data suggest that SMRT, when it is recruited to a promoter by direct DNA binding or through association with an unliganded receptor, functions as a potent transcriptional repressor.

In conclusion, we have identified a novel co-repressor which mediates the transcriptional silencing of RAR and TR. Transcriptional silencing has been shown to be important in development, cell differentiation and in the oncogenic activity of *v-erbA*^{1,3}. In fact, *v-erbA* mutants that harbour the corresponding TR point mutation at position 160 neither suppress basal transcription nor are capable of oncogenic transformation^{14,15}. The function of SMRT as a silencing mediator of RAR and TR is analogous to several co-repressors in other systems^{21,25}. Because GAL-SMRT functions as a potent repressor when bound to DNA, the function of unliganded receptors may be to bring SMRT with them to target promoters via protein-protein interaction. Thus, the repressor function of TR and RAR involves direct interaction with SMRT, possibly in a way that stabilizes or promotes their interaction with the transcription factor TFIIB^{26,28}. We have shown that ligand causes the dissociation of SMRT from the receptor, triggering the activation process. This could be followed (or be coincident) with an induced conformational change in the C-terminal trans-activation domain (rc, also called AF-2), allowing association with co-activators²⁹. Thus, as we have previously suggested¹⁵, the ligand-dependent activation of TR would represent two separable processes, including relief of repression and net activation. The isolation of SMRT now provides a tool for dissecting the molecular basis of *trans*-repression by nuclear hormone receptors.

Note added in proof: In an accompanying paper, Hörlein *et al.*³¹ propose the name TRAC (for thyroid-, retinoic acid-receptor-associated co-repressor) for the new family of proteins. □

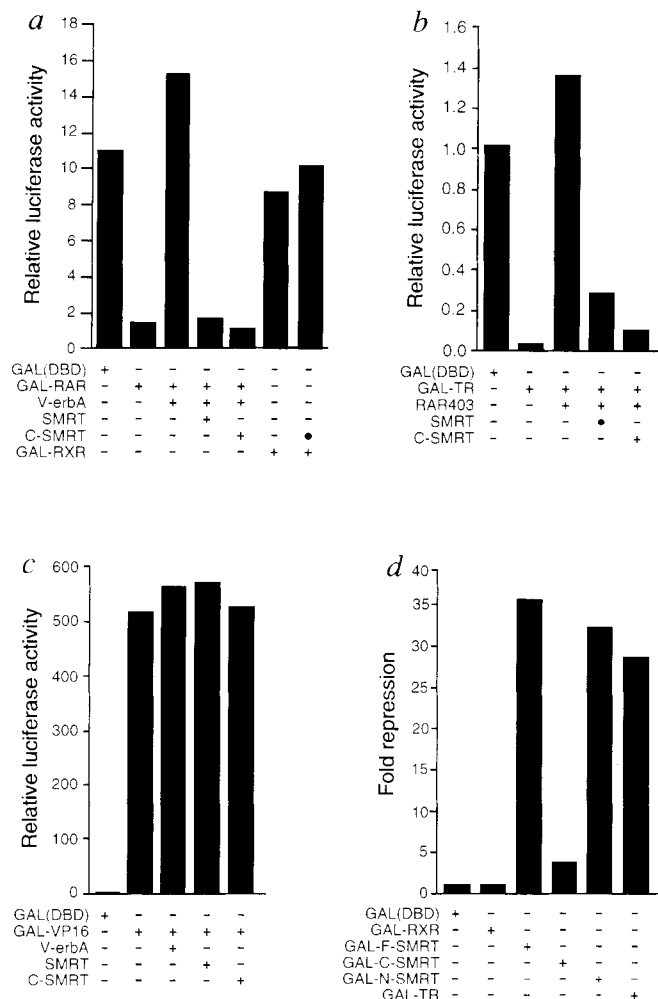


FIG. 4 Mediation of the silencing effect of hRAR α and hTR β by SMRT *in vivo*. **a**, Effect of v-ErbA and SMRT on the basal level promoter activity of GAL-RAR (GAL4 DBD-hRAR α 156–462) and GAL-RXR (GAL4 DBD fusion of hRXR α LBD). **b**, Effect of RAR403 truncation mutant and SMRT on the basal-level promoter activity of GAL-TR (GAL4 DBD-hTR β 173–456). **c**, v-ErbA and full-length SMRT or C-SMRT have no effect on GAL-VP16 activity. **d**, The intrinsic *trans*-repression domain of SMRT analysed by GAL4 DBD fusions. Fold repression was calculated by dividing the normalized luciferase activity transfected with the GAL4 DBD alone by those transfected with 30 ng GAL DBD fusion constructs. GAL-F-SMRT, GAL4 DBD fusion of full-length SMRT; GAL-N-SMRT, GAL4 DBD fusion of N terminus of SMRT (aa 1–981); GAL-C-SMRT, GAL4 DBD fusion of C-SMRT; GAL-TR, GAL4 DBD fusion of hTR β LBD.

METHODS. Transient transfection assay. The CV-1 cells were plated in 24-well plates at a density of 50,000 cells per well. The expression plasmids were transfected into cells by lipofection. In each transfection, 5 ng GAL-RAR and 15 ng *v-erbA* or SMRT were used together with 150 ng reporter construct containing 4 copies of GAL4 binding sites in front of a minimal thymidine-kinase promoter and a CMX- β -gal construct as an internal control. The relative luciferase activity was calculated by normalizing to the β -gal activity.

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Market analysis

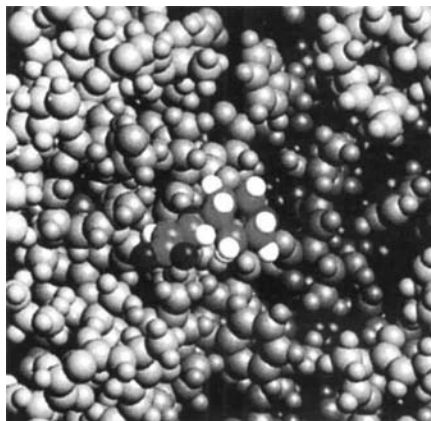
New items on the market this issue include an uninterruptible power supply, a biomedical interlaboratory service, an electrochemistry labstation, scientific cameras, capillary electrophoresis accessories and solvent handling equipment.

FOR synthesis automation Advanced Chemtech offers a new instrument for multiple organic synthesis (*Reader Service No. 100*). The model 496 **multiple organic synthesizer** (MOS) is designed for solid-phase synthesis and offers capabilities such as reaction heating and cooling, environmentally controlled reactions and multispeed vortex mixing. The manufacturer states that the instrument can prepare 96 different compounds in a single run, allowing different protocols to be carried out independently in each reaction vessel. A user-controlled, three-stage reaction environment features fully inert reagent and monomer vessels, an inert reaction block and provides a nitrogen or argon blanket to the entire reaction area. The model 496 can provide reactor temperatures from -70 to 150°C with equalization of up to 10°C per minute. Additionally, its multiple-line, waste-disposal system enables segregation of table-top waste for disposal classification and reagent recovery. This is a Windows-driven system that allows the design of protocols from start to finish.

The new five-gallon EM ReCycle system from EM Science helps laboratories avoid **solvent disposal** problems (*Reader Service No. 101*). The Baby Bulk 18.5-l container saves money by eliminating the need for triple rinsing and disposal of small non-recyclable bottles, and a positive-pressure dispensing system provides constant, easy-to-control flow. It features volume indicators that let laboratory personnel monitor solvent levels easily. Its size enables companies to dedicate individual closed-system drums to each laboratory, assuring content purity. Other features include liquid quick connect SS-QT4-, inert-gas quick connect SS-QT2-, relief and shut-off valves and an impact-resistant stainless steel construction for maximum durability. The five-gallon container meets NFPA volume limits for flammables in the laboratory and features a 15-psig working pressure. Several grades of HPLC and biosynthesis solvents can be ordered in the new container including acetone, acetonitrile, ethyl acetate, methanol, dichloromethane and *n*-hexane (85 per cent).

Software

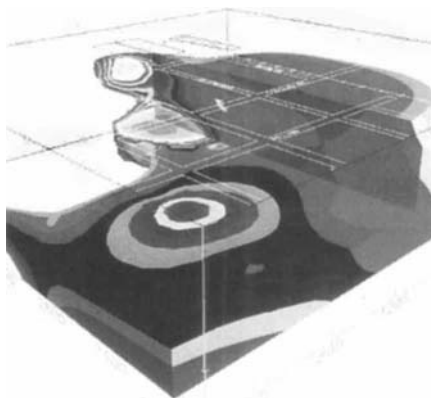
Chemical Design has developed Chem-X, a **high-throughput screening approach to lead generation** (*Reader Service No. 102*). The manufacturer states that this program rapidly selects multiple compounds from large databases to match their ability to dock in a particular receptor. The program is designed to identify the ways in which small



Select compounds from large databases using the Chem-X lead generation software.

molecule ligands may interact at the receptor site. Each interaction model is stored as a receptor pharmacophore in a database, which may then be used as queries to search a three-dimensional database or as templates for *de novo* molecule building.

Groundwater Technology is using three-dimensional graphic imaging technology to help its customers **visualize soil and groundwater contamination** in subsurface geologies (*Reader Service No. 103*). The Earthvision software is designed to allow better informed decisions to be made about contamination cleanup options. It should



Earthvision for visualizing contamination.

also prove useful for demonstrating cleanup to regulators and to contribute information to legal disputes over responsibility for contamination at industrial sites. The program gives a three-dimensional representation to the

charts, graphs and numerical data used to describe the concentration and distribution of soil and groundwater contamination. The Earthvision software utilized by the company depicts the depth, width and concentration of contamination by comparing several models over time.

Megalon has announced additions to its product line with three new Windows-based **scientific software packages**: ChemStructure, Compounds and Unistat 3.0 (*Reader Service No. 104*). ChemStructure is designed to be a high-performance program for chemical and structural formulae, schematics and experimental setups. Structures can be drawn and developed on-screen, then printed or exported to layout programs or word processors. It has a comprehensive range of bonds; fast access to frequently used partial



Megalon scientific software package.

structures; a graphical objects library; high-level drawing aids; over 51 adjustable and rotatable fonts, including special characters; graphic design tools; and import and export capabilities to other applications, including Alchemy, Moby, Sybyl, and Isis/Draw. The manufacturer claims that Compounds is a single source compound encyclopaedia with physical properties and spectral data for approximately 27,000 organic compounds, and chemical structures for 16,000 compounds. It allows compound identity to be established based on a variety of spectral comparisons including IR, ultraviolet/visible, ^1H -NMR, ^{13}C -NMR, Raman and mass spectra, which are all included in the package. Unistat 3.0 is said to provide a comprehensive set of functions for the handling, analysis and presentation of statistical data within the Windows environment. It provides a statistics-oriented spreadsheet and data visualization tools, including fully scaled, annotated graphs, two- and three-dimensional scatter diagrams and spin plots. Data can be exchanged with Lotus 1-2-3, Excel and dBase files.

Marie is a Window-based, **stock-control system** for radiological safety officers (*Reader Service No. 105*). LabLogic has collaborated with licensed users to design a package that provides simplified record keeping to comply with holding, disposal and accumulation licenses. An on-screen warning is automati-

cally given when limits are approached, and an audit trail is logged for any alterations. It is possible to have an accurate up-to-date status of stock at any time, thus satisfying the rigorous demands of regulatory authorities and minimizing disposal costs.

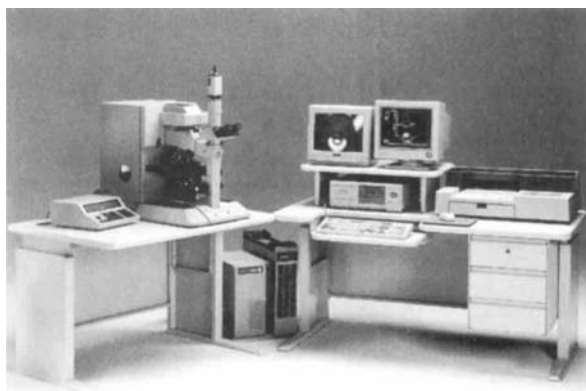
CE

For capillary electrophoresis (CE) Hewlett-Packard has introduced **CE capillaries** containing a permanently adsorbed layer of polyvinyl alcohol (PVA) (*Reader Service No. 106*). Because the inside walls of the capillaries are coated with polyvinyl alcohol, the capillaries can permanently shield out negatively charged silanol groups. This is designed to provide a capillary with virtually no electroosmotic flow over a wide pH range, from pH 2.5–9.5. This is said to eliminate much of the peak tailing found with the separation of many proteins and amines on uncoated capillaries. Applications include protein analysis at physiological pH, isoelectric focusing, pharmaceutical and ion analysis. The PVA coating is available in standard capillaries and extended light-path capillaries for high-sensitivity applications.

Thermo Separation Products has introduced a **chiral chemistry kit** containing chiral selectors for analysis of enantiomers by capillary electrophoresis (CE) (*Reader Service No. 107*). The manufacturer states that increasing interest in CE as an alternative technique for separation of chiral compounds because of its efficiency, low per-analysis cost and short analysis time. The kit comprises chiral selectors for the main classes of chiral separations described in the literature. It also includes suitable standards of racemic mixtures for rapid validation of the selected techniques. It is said to provide uncomplicated access to chiral separations with a method-development flow chart, detailed instructions with examples of standard separations, and an introduction textbook.

Imaging

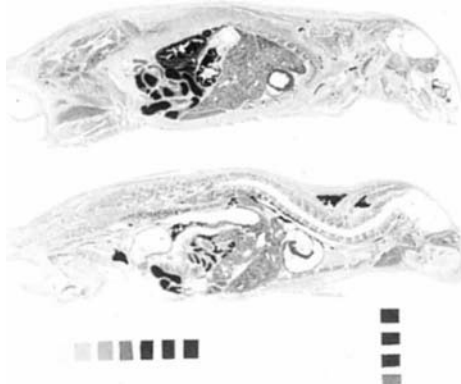
Spectra-Tech has introduced the **IR μ s, infrared microanalysis system** (*Reader Service No. 108*). The system combines optical microscopy and infrared spectroscopy with dedicated Windows-based software. It allows the user to determine the chemical and molecular properties of a wide variety of



IR μ s optical microscopy and IR-spectroscopy system.

organic and inorganic materials. Samples as small as 10 μ m can be measured. The instrument should prove useful for analysing the chemical structure of multilayer films, pharmaceuticals, contaminants, biological samples, forensic evidence and surface coatings. A separate data acquisition computer and hard disk allow the user to collect data while using the independent workstation for other tasks, without reducing data acquisition or mapping speed. The system allows the user to analyse samples in transmission and reflectance modes along with the options for performing ATR surface analysis, grazing angle surface analysis and molecular mapping function.

Inquiry is a software program designed to assist pharmaceutical researchers in the evaluation of **whole body autoradiography** images produced by the Molecular



Molecular Dynamics' for autoradiograph.

Dynamics PhosphorImager and densitometer systems (*Reader Service No. 109*). The program offers image enhancement, list-directed sampling and contour tracking with point-and-click quantitations. An audit trail is generated during use for good laboratory practice documentation. System counts and film optical densities can be standardized to μ Cu per gram or disintegrations per minute to enable accurate quantitative comparisons between experiments. Applications include metabolic glucose utilization and blood-flow mapping studies.

Data Cell is now offering Photometrics' cooled, scientific **CCD cameras** (*Reader Service No. 110*). These cameras can be used for low-light and high-light levels, as well as high dynamic range applications. The S200 family of HCCD cameras uses from medium- to high-resolution sensors (2,048 $\times$ 2,048), with 12-, 14- or 16-bit digitization. The PXL series is said to be a powerful HCCD camera, capable of high frame rates and variable speed operation. The PXL series also supports high-resolution sensors (up to 4,096 $\times$ 4,096), with

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pixel binning and regional readout for even faster frame (+100). These should prove useful for many applications including astronomy, microscopy, forensics, medical imaging, fluorescence *in situ* hybridization, calcium ratio imaging, cloud cover analysis, X-ray imaging, spectroscopy, as well as semiconductor failure analysis and precision metrology. The cooling options range from air-cooled, three-stage Peltier to cryogenic cooling.

Spectral Diagnostics has announced the release of its new **spectral bioimaging system** called SpectralCube (*Reader Service No. 111*). The system combines proprietary Fourier transform imaging spectroscopy with digital image and spectral processing. This is designed to produce simultaneous spectroscopy at each of the tens of thousands of



Spectral Diagnostics' bioimaging system.

pixels. The manufacturer states that the system has combined chemical spectroscopy and imaging for fluorescence or transmission microscopy. This incorporates the digital image with the internal chemistry or physics of the cell or tissue. The system may prove useful for mapping molecular or genetic structures.

Electrochemistry

The VoltaLab 32 **electrochemical laboratory** has been designed to meet the needs of researchers involved in fundamental electrochemistry (*Reader Service No. 112*). The system has a single generator and potentiostat, which enables voltages and amperages to be monitored efficiently in applications as diverse as electrosynthesis, electrochemical process and corrosion studies, battery performance and new material testing. Control is simplified by the Windows-based VoltaMaster 2.0 software. The unit features the IMT101 electrochemical interface, a programmable signal generator driving a built-in 33 V/2 A potentiostat/galvanostat that enables current ranges from 25 nA to 2 A to be monitored, with sensitivity as low as 12.5 pA for current and 1 mV for potential. In addition, features such as the determination of ohmic drop compensation, applicable in high- and low-conductivity media for both strong and weak currents, are designed to eliminate the need to use an oscilloscope. For higher current requirements up to 50 A, an adapter and software upgrade are avail-

able — higher potentials or current can be accommodated with a customized VoltLab. For ultrasonic treatment of liquid chemical and biological samples, Labcaire Systems offers the Microson XL2007 (*Reader Service No. 113*). This high-intensity **ultrasonic cell disrupter** is designed specifically for processing small sample volumes. It is equipped with a 100 W generator and new circuitry for demanding applications. The small-diameter probes of the instrument are designed to allow for processing of samples, from 200 μ l to 100 ml. Specially designed electronics are used to deliver more power to the probe tip. The unit also features load monitoring, automatic tuning, a digital wattmeter with LCD display and a hand-held probe for easy operation. An optional footswitch allows for manual pulsing and hands-free operation.

Gould has introduced the WindoGraf **electrophysiology monitor**, a portable, four-channel recorder, which should prove suitable for operations such as intracardiac device implants (*Reader Service No. 114*). The unit weighs only 20.5 kg with signal conditioners, and its small size (30.5 cm $\times$ 43 cm $\times$ 30.5 cm) and portability enable it to be used in cramped space. ECG and other waveforms can be displayed in real time on the unit's built-in display, while a thermal-array recorder produces high-resolution, hard-copy output on 12.0-cm wide fanfold paper. Heart rate and time inter-



The WindoGraf electrophysiology monitor.

vals can be shown numerically on a four-digit LED display on the plug-in ECG/Biotach signal conditioner module. The instrument features user-selectable, horizontal- or vertical-trace presentations. On-screen cursors allow the measurement of cycle lengths, which can be printed along with waveforms on the recorder chart. The sample rate in the normal recording mode is 800 samples per second per channel, but it can be user-specified up to 10,000 samples per second. The hard drive can store 4 h 45 min of continuous data.

Sundry items

Köttermann has developed the System Laboratory, a new **laboratory furniture setup** to accommodate new concepts in efficiency and space economy (*Reader Service No. 115*). In addition to a traditional linear arrangement, the system uses octagonal

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NEW! Severn Biotech Limited has been appointed as the exclusive **U.K.** distributor for Takara Japan from June 1995. Takara manufactures a range of Molecular Biology and Cell Biology products to include Restriction Enzymes, Modifying Enzymes, Sequencing Kits and a whole spectrum of Bio-Molecular products for research. Please contact SBL's offices for further details and a price catalogue.

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structures, work islands, individual and special workstations. The central element of this system is the triangular media supply column, which is designed to allow all media to be available in a vertical arrangement. It can be used in a variety of ways to allow access — even when large equipment is in use.

The LR series of intelligent **pen recorders**, which combine universal input capabilities with powerful measurement capabilities, is now available from Marton Instruments in versions from 1–12 pens (*Reader Service No. 116*). The recorders can be supplied in upright or flatbed form and offer full digital measurement and display capability, in addition to the analog pen recording. Each channel is provided with a 20-character vacuum-fluorescent display, which gives an accurate digital indication of each incoming signal,



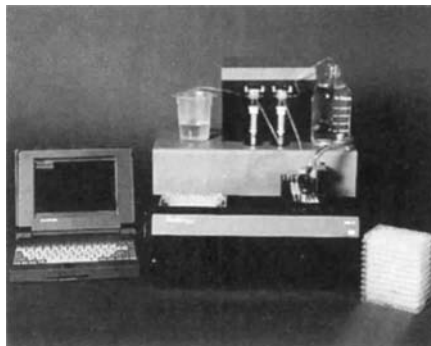
Marton Instruments' LR series recorders.

including unit marks. Incoming voltage signals can be scaled into engineering units, and can be displayed independently in a wider range than that selected by the user for hard-copy recording. Mathematical functions can also be included to account for nonlinear relationships, or to combine different measured inputs into a single recorded trace.

The global **biomedical interlaboratory service** has been introduced by LabLink to facilitate the exchange of laboratory produced specimens through a published periodic directory (*Reader Service No. 117*). Investigators should now be able to announce the conditional or unconditional availability of any physical samples developed in their laboratories — synthetic or purified compounds, antibodies, enzymes, genetic vectors or recombinant DNA, microbial strains or cell lines, even transgenic animals. Directory listings will appear in the form of short self-created abstracts that describe the materials offered or desired, provide the name and address of a contact person, and optionally, define any specific interest in collaborative studies or the cost for product supply, and indicate any relevant publications. url: <http://magibox.net/~lablink/lablinkhome.html>

The TitertekPlus MRD8 is a high-throughput **microplate processing device**, designed for enzyme-linked immunoassays, plate coating

and media exchange applications (*Reader Service No. 118*). Incorporating either ICN's patented manifold system or the company's four-in-one syringe, the MRD8 has a working range of 5 μ l to 10 ml with CVs less than one per cent. The optional S20 plate stacker allows 20 microplates to be processed without user intervention. The 20-plate maga-



ICN's MRD8 microplate processing device.

zines are compatible with other Titertek microplate devices, such as photometers, washers, dispensers and the QuadFlex sample processor. The media exchange option for cell-based assays performs a gentle aspiration and incorporates dispense heads designed to minimize disruption of the cell layer. Fully programmable, the MRD8 provides flexibility in assay design with user definition of wash cycles, soak times, reagent additions, an so on.

The Electric Power Research Institute and Precise Power have combined to offer an **uninterruptible power supply** to provide power in case of a utility interruption (*Reader Service No. 119*). This was done by combining the Written-Pole, large-horsepower, single-phase motor with a generator allowing for a single-phase line. The manufacturer states that this system is cleaner because the generator power eliminates transient, voltage sags and surges that cause equipment interruptions. Harmonic feedback to the utility is also eliminated. The output can be both single-phase or three-phase.

Brinkmann Instruments offers its 1995 **product catalogue** (*Reader Service No. 120*). This full-colour, 56-page brochure presents a wide variety of laboratory and industrial products for research, quality control, manufacturing process, environmental monitoring and education. Products featured include the full line of Eppendorf liquid-handling products, Brinkmann Polytron homogenizers, overhead mixers, sample preparation equipment, Lauda baths and chillers, Buchi rotary evaporators and accessories. □

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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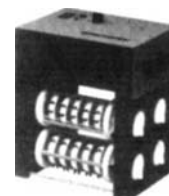
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